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CAIO SÉRGIO SANTOS

MICROBIOTA AERÓBIA DO SÊMEN E DA MUCOSA PREPUCIAL E EFEITO DOS ANTIMICROBIANOS NA CONSERVAÇÃO DOS PARÂMETROS ESPERMÁTICOS DE CATETOS (*Pecari tajacu* LINNAEUS, 1758) EM CATIVEIRO

MOSSORÓ
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Tese apresentada ao Doutorado em Ciência Animal do Programa de Pós-Graduação em Ciência Animal da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Doutor em Ciência Animal.

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal

Orientador: Alexandre Rodrigues Silva, Prof. Dr.

Co-orientador: Francisco Marlon Carneiro Feijó, Prof. Dr.

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À minha mãe, Maria Antônia da Conceição,
e a minha tia Lúcia Pereira, pelo amor e cuidado

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“A dúvida é o princípio da sabedoria”

Aristóteles

RESUMO

A presença de bactérias no sêmen pode causar perdas na função espermática e, consequentemente, interferir nas biotecnologias reprodutivas que façam uso desse material biológico. Dito isso, objetivou-se descrever a microbiota bacteriana aeróbia do sêmen e prepúcio dos catetos (*Pecari tajacu*) criados em cativeiro, bem como o impacto do uso de substâncias antimicrobianas na carga bacteriana e função espermática durante a conservação do sêmen dessa espécie. No primeiro experimento, foi realizada a cultura das bactérias aeróbias em amostras de sêmen e prepúcio de nove animais, bem como a quantificação destas e avaliação de parâmetros espermáticos no sêmen. Os isolados foram identificados e testados frente a concentrações de penicilina-estreptomicina, gentamicina e do gel da *Aloe vera*. *Corynebacterium* spp. e *Staphylococcus* spp. foram isolados em maior número no sêmen (64,1% e 20,5%, respectivamente) e no prepúcio (60,6% e 24,2%, respectivamente), variando de 0,4 a 21 × 10^5 unidades formadoras de colônia (UFC) por mililitro. A carga de *Corynebacterium* spp. foi correlacionada negativamente ($P < 0,05$) com a integridade da membrana espermática ($r = -0.73055$) e velocidade curvilinear ($r = -0.69048$). A combinação penicilina-estreptomicina (PE) e gentamicina (G) inibiram a maioria dos microrganismos enquanto a *A. vera* demonstrou baixa atividade antimicrobiana. No segundo experimento, foi analisada a toxicidade de antimicrobianos sobre a longevidade espermática pelo teste de termo resistência em seis amostras de sêmen, sendo cada uma diluída em Tris (controle) e em Tris acrescido da combinação penicilina-estreptomicina (2000 UI/mL-2 mg/mL) ou de gentamicina (70 µg/mL), e mantidas por 180 min a 37 °C. Verificou-se que os tratamentos não diferiram ($P > 0,05$) até 180 min, mas que o tratamento contendo G reduziu ($P < 0,05$) a integridade da membrana e a atividade mitocondrial das células espermáticas aos 180 min (53,1 ± 7,1% e 50,7 ± 6,2%, respectivamente) se comparado a 0 min (80,5 ± 4,7% e 86,3 ± 3,4%, respectivamente). No terceiro experimento, foi avaliado o efeito de duas concentrações de PE (2000 UI/mL – 2 mg/mL [2] e 1000 UI/mL – 1 mg/mL [1]) e G (70 µg/mL [7] e 30 µg/mL [3]) adicionadas aos diluentes Tris + 20% de gema de ovo (TG) e Tris + 20% de *A. vera* (TA) sobre a carga bacteriana e qualidade espermática em 10 amostras de sêmen mantidas sob refrigeração (5 °C) até 36 h. O tratamento contendo PE2, PE1 e G7, independente do diluente, controlaram ($P < 0,05$) o crescimento bacteriano durante o armazenamento (variando de 0.5 ± 0.3 10^3 a 10 ± 4.1 x 10^3 UFC/mL) até 36 h. Os tratamentos diluídos com TG com qualquer um dos antimicrobianos não demonstraram diferenças ($P > 0,05$) para a carga bacteriana e parâmetros espermáticos entre 0 e 36 h. Os tratamentos com TA, com ou sem antimicrobianos, afetaram ($P < 0,05$) a integridade da membrana e atividade mitocondrial dos espermatozoides após 12 h. O tratamento TG-G7 se destacou por manter algumas variáveis espermáticas por mais tempo, como a motilidade total (41,9 ± 6,1%) e progressiva (15 ± 2,6%) até 24 h, bem como a integridade de membrana (58.3 ± 2.1%) e velocidade curvilínea (76.7 ± 5.8%) até 36 h. Diante dos resultados, demonstrou-se a ocorrência de bactérias contaminantes no sêmen e prepúcio de catetos criados em cativeiro, com destaque para *Corynebacterium* spp. e *Staphylococcus* spp., bem como um impacto negativo da primeira sobre a função espermática no sêmen fresco. A combinação penicilina-estreptomicina (2000 UI/mL-2 mg/mL) e gentamicina (70 µg/mL) podem ser adicionadas ao Tris na diluição de sêmen, incubado a 37 °C por até 120 min, sem causar efeitos tóxicos aos espermatozoides. Estas drogas também foram eficazes no controle das bactérias presentes no sêmen refrigerado destes animais, por até 36 h, sem causar prejuizo a longevidade espermática.

Palavras-chave: *Aloe vera*; bactérias; refrigeração; termo-resistência.

ABSTRACT

The presence of bacteria in the semen can cause losses in sperm function and, consequently, interfere with reproductive biotechnologies that make use of this biological material. That said, the objective was to describe the aerobic bacterial microbiota of captive collared peccaries (*Pecari tajacu*) semen and foreskin, as well as the impact of the use of antimicrobial substances on the bacterial load and sperm function during the semen conservation of this species. In the first experiment, aerobic bacteria were cultured in semen and foreskin samples from nine animals, as well as their quantification and evaluation of sperm parameters in the semen. The isolates were identified and tested against concentrations of penicillin-streptomycin, gentamicin and *Aloe vera* gel. *Corynebacterium* spp. and *Staphylococcus* spp. were isolated in greater numbers in semen (64.1% and 20.5%, respectively) and in the foreskin (60.6% and 24.2%, respectively), ranging from 0.4 to 21 × 10^5 colony-forming units (CFU) per milliliter. The load of *Corynebacterium* spp. it was negatively correlated ($P < 0.05$) with the integrity of the sperm membrane ($r = -0.73055$) and curvilinear velocity ($r = -0.69048$). The combination of streptomycin-penicillin (SP) and gentamicin (G) inhibited the majority of microorganisms and *A. vera* demonstrated a weak antimicrobial potential. In the second experiment, the toxicity of antimicrobials on sperm longevity was analyzed by the thermoresistance test in six semen samples, each diluted in Tris alone (control) and Tris plus the streptomycin-penicillin combination (2 mg/mL - 2000 IU/mL) or gentamicin (70 µg/mL), and maintained for 180 min at 37 °C. It was found that the treatments did not differ ($P > 0.05$) until 180 min, but that the treatment containing G reduced ($P < 0.05$) the sperm membrane integrity and mitochondrial activity at 180 min (53, 1 ± 7.1% and 50.7 ± 6.2%, respectively) if compared to 0 min (80.5 ± 4.7% and 86.3 ± 3.4%, respectively). In the third experiment, the effect of two concentrations of SP (2 mg/mL - 2000 IU/mL [2] and 1 mg/mL - 1000 IU/mL [1]) and G (70 µg/mL [7] and 30 µg/mL [3]) added to Tris + 20% egg yolk (TE) and Tris + 20% *A. vera* (TA) diluents on bacterial load and sperm quality in 10 semen samples kept under refrigeration (5 ° C) up to 36 h. The treatment containing SP2, SP1 and G7, regardless of the diluent, controlled ($P < 0.05$) the bacterial growth during storage (ranging from 0.5 ± 0.3 10^3 to 10 ± 4.1 x 10^3 CFU/mL) up to 36 h. Treatments diluted with TE with any of the antimicrobials showed no differences ($P > 0.05$) for bacterial load and sperm parameters between 0 and 36 h. TA treatments, with or without antimicrobials, affected ($P < 0.05$) the sperm membrane integrity and mitochondrial activity after 12 h. The TE-G7 treatment stood out for maintaining some sperm variables for a longer time, such as total motility (41.9 ± 6.1%) and progressive (15 ± 2.6%) up to 24 h, as well as membrane integrity (58.3 ± 2.1%) and curvilinear velocity (76.7 ± 5.8%) up to 36 h. Given the results, it was demonstrated the occurrence of bacterial contaminants in semen and foreskin peccaries bred in captivity, especially *Corynebacterium* spp. and *Staphylococcus* spp., as well as a negative impact of the first on sperm function in fresh semen. The streptomycin-penicillin combination (2 mg/mL - 2000 IU/mL) and gentamicin (70 µg/mL) can be added to Tris in the semen dilution, incubated at 37 °C for up to 120 min, without causing toxic effects to sperm. These drugs were also effective in controlling the bacteria present in the refrigerated semen of these animals, for up to 36 h, without affecting sperm longevity.

Keywords: *Aloe vera*; bacteria; refrigeration; thermoresistance.

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LISTA DE ABREVIATURAS E SIGLAS

RNA	ácido ribonucleico
ATP	adenosine thiphosphate
ALH	amplitude of lateral head
AMP	antimicrobial petides
AI	artificial insemination
ART	Assisted Reproductive Technique
VAP	average path velocity
BCF	beat cross frequency
CFDA	carboxyfluorescein diacetate
cm	centímetro
CEMAS	Centro de Multiplicação de Animais Silvestres
r	coeficiente de correlação
CFU/ml	colony forming units per mililitre
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
VCL	curvilinear velocity
spp.	espécies
EEC	European Economic Comunity
PROC GLM	General Linear Models Procedure
G3	gentamicin 30 µg/mL
G7	gentamicin 70 µg/mL
GTLS	gentamicin-tylosin-lincomycin-spectinomycin
°C	graus Celsius
H342	Hoechst 342
h	hora
HIF1a	hypoxia inducer factor 1 alpha
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
IU/mL	internacional units per mililitre
LAMIV	Laboratório de Microbiologia Veternária

LIN	linearity
Log_{10}	logaritmo 10
m	metro
m^2	metro quadrado
µg/mL	micrograma por mililitro
µl	microlitro
µm	micrometro
µm/s	micrometro por segundo
10^6	milhões
mg/mL	miligrama por mililitro
mM	milimolar
mOsm/mL	miliosmol pos mililitro
MIC	minimal inhibition concentration
MBC	minimal bactericidal concentration
min	minuto
CMXRos	Mito Tracker® Red
MH agar	Müller-Hinton agar
MH broth	Müller-Hinton broth
PBD-1	porcine beta-defensin 1
PBD-2	porcine beta-defensin 2
PMAP-36	porcine myeloid antimicrobial peptide 36
PMAP-37	porcine myeloid antimicrobial peptides
pH	potencial hidrogeniônico
P	probabilidade de significância
PM	progressive motility
PR-39	proline-arginine-rich antimicrobial peptide
PI	propidium iodide
ROS	reactive oxygen species
s	segundo
SLC	single layer centrigugation
SEM	standard error mean
VSL	straight line velocity
STR	straightness

SP1	streptomycin-penicillin 1 mg/mL – 1000 IU/mL
SP2	streptomycin-penicillin 2 mg/mL – 2000 IU/mL
TM	total motility
TA	Tris-*Aloe vera*
TE	Tris-egg yolk
UFERSA	Universidade Federal Rural do Semi-Árido
V	volt

LISTA DE SÍMBOLOS

©	Copyright
>	Maior que
±	Mais ou menos
®	Marca registrada
≤	Menor ou igual
<	Menor que
%	Porcentagem
√	Raíz quadrada
™	Trademark

SUMÁRIO

1 INTRODUÇÃO

O *Pecari tajacu* (Linnaeus, 1758), conhecido popularmente como cateto ou caititu, é uma espécie de mamífero selvagem amplamente distribuída no continente americano, habitando regiões desde o sul dos Estados Unidos até o norte da Argentina, incluindo países como o México, Colômbia, Peru, Equador, Brasil, Venezuela, Guianas, Suriname, Bolívia e Paraguai. Suas populações podem ser encontradas nos mais variados *habitats* como bosques, florestas tropicais e de altitude, savanas e desertos, apresentado importância ecológica como dispersores de sementes e servindo de presa para grandes carnívoros (GONGORA et al., 2011).

Apresenta também importância econômica, com uma carne de alta palatabilidade além do valor agregado da sua pele para a fabricação de artigos de couro destinados ao mercado internacional (SANTOS et al., 2009). Apesar da caça para subsistência ser permitida para famílias rurais, o valor de mercado agregado à carne e à pele torna frequente a caça ilegal desse animal silvestre (BODMER et al., 2004). Este fato, atrelado ao fator de perda e destruição do *habitat* e fragmentação dos locais de ocupação (DESBIEZ et al., 2012), afetam negativamente o número de indivíduos das suas populações. Dentre os biomas brasileiros de ocorrência dos catetos, a Mata Atlântica e a Caatinga apresentam-se como alguns dos quais estão bastante degradados e cujos problemas na conservação de espécies remanescentes são bastante preocupantes (BEISIEGEL et al., 2012).

Nesse contexto, estratégias de conservação podem ser desenvolvidas, as quais caracterizam-se por medidas *in situ*, em que os animais são mantidos em seu ambiente natural; *ex situ in vivo*, em que o animal é mantido fora do seu ambiente; e *ex situ in vitro*, por meio da conservação do seu germoplasma (COSTA; MARTINS, 2008). A espécie em estudo tem sido foco de estudos recentes com o propósito de aperfeiçoar as técnicas conservação do sêmen desses animais por meio da criopreservação (CASTELO et al., 2010; SILVA et al., 2013; CAMPOS et al., 2014; SOUZA et al., 2015). Além disso, Souza et al. (2016) relataram a possibilidade de armazenar os gametas masculinos dos catetos sob refrigeração a 5 °C por até 36 h, utilizando o diluente Tris acrescido de dois crioprotetores externos, a gema de ovo ou a *A. vera*. Nesse estudo foi verificado que a refrigeração do sêmen de catetos foi bem sucedida utilizando 20% de gema de ovo ou 20% de gel de *A. vera* durante o armazenamento por 36 h.

Entretanto, existem riscos sanitários associados a tecnologia de sêmen, a qual apresenta potencial para disseminação de doenças infecciosas (EAGLESOME; GARCIA, 1997) por meio da inseminação artificial. Os patógenos podem vir a contaminar o sêmen durante a coleta e o processamento do mesmo, caso não sejam adotadas boas práticas (LOPEZ RODRIGUEZ et al., 2017). Adicionalmente, essa contaminação pode advir de infecções sistêmicas prévias, bem como de infecções nos sistemas urinário e reprodutor e do contato do ejaculado com secreções prepuciais (FERNANDES, 2012).

A despeito do que foi relatado, o estudo da microbiota do trato reprodutivo é relevante para o aperfeiçoamento das técnicas de conservação de sêmen. Mas, diferente de estudos sobre a microbiota da pele (STRUBLE et al., 2018) e do trato gastrointestinal (BURBACH et al., 2017), os estudos destinados a caracterizar as bactérias do trato genitourinário são escassos em humanos (BAUD et al., 2019), animais domésticos (ALTHOUSE; LU, 2005; MORENO et al., 2016) e selvagens (GHONEIM et al., 2014; JOHNSTON et al., 1998).

É importante ressaltar que a presença destes microrganismos no sêmen pode causar danos ao espermatozoide, dependendo do seu tipo e quantidade (KUSTER; ALTHOUSE, 2016; BONET et al., 2018), o que afeta negativamente a qualidade do sêmen destinado a refrigeração. Por estes motivos, durante os protocolos de conservação de sêmen são, geralmente, adicionados antimicrobianos aos diluentes como forma de reduzirr a multiplicação bacteriana (MORRELL; WALLGREN, 2011), não sendo relatada a inclusão destes nos diluentes seminais de catetos. Em contrapartida, algumas drogas podem vir a afetar a sobrevivência dos espermatozoides, sendo por vezes restrita a escolha dos princípios ativos. Além disso, o uso indiscriminado de antimicrobianos pode acarretar em resistência bacteriana, mesmo quando adotadas pequenas quantidades (MORRELL; WALLGREN, 2011; FERNANDES, 2012).

Com isso, a problemática da resistência aos antimicrobianos gera demanda de estudos sobre novas combinações de antibióticos com elevada atividade antibacteriana e efeitos não significativos sobre os espermatozoides (GLORIA et al., 2014). Outro viés de combate a resistência é a busca de novos agentes bactericidas, que podem ser oriundos de produtos naturais bioativos (SILVEIRA et al., 2006), como fitoterápicos e outros compostos obtidos de plantas. Nesse aspecto, uma alternativa para o controle do crescimento bacteriano na conservação do sêmen de catetos seria o gel da *Aloe vera*, cuja fração não volátil apresenta atividade antibacteriana (MAAN et al., 2018; KUMAR et al., 2019), e que demonstrou ser um eficaz crioprotetor não-penetrante durante a refrigeração de sêmen na espécie (SOUZA et al., 2016), porém não sendo utilizado como antimicrobiano na composição do diluente.

Diante do que foi exposto, a caracterização da microbiota reprodutiva de catetos e seu perfil de sensibilidade a antimicrobianos utilizados em diluentes de sêmen poderia ajudar na escolha de protocolos adequados para conservação desse material. Observa-se também a necessidade de estudos que averiguem os efeitos das substâncias antimicrobianas adicionados aos diluentes de sêmen sobre a carga bacteriana e função espermática durante sua conservação. Para tanto, podem ser utilizados antibacterianos usuais e alternativos, como o gel da *A. vera*, visto que seu potencial antimicrobiano é desconhecido para a espécie e que promoveu uma adequada proteção ao frio durante a refrigeração do sêmen dos catetos.

2 JUSTIFICATIVA

Os estudos direcionados a conservação de germoplasma de diversos animais silvestres da Caatinga no Brasil vêm sendo desenvolvidos nos últimos anos, motivados por problemas ambientais que ameaçam às espécies da fauna local. Um exemplo é o cateto, cuja população vem reduzindo no decorrer dos últimos anos devido a ação antrópica sobre seu *habitat*, causando perda e fragmentação dessas áreas. A maioria dos protocolos de conservação de sêmen de animais silvestres são baseados em protocolos prévios já utilizados em animais domésticos. Estes, entretanto, diferem entre espécies, sejam elas domésticas ou silvestres, visto que o mesmo protocolo pode promover a conservação do sêmen de uma espécie, mantendo sua qualidade espermática, ou interferir nos parâmetros espermáticos de outra espécie, como motilidade e viabilidade, influenciando negativamente na qualidade seminal.

Na tecnologia de sêmen, os problemas de contaminação bacteriana são frequentes, visto que é impossível uma colheita totalmente estéril. O sêmen pode se contaminar ao sair pela uretra e entrar em contato com o ambiente externo, bem como durante sua manipulação e processamento. Dependendo do tipo bacteriano e da sua concentração, algumas funções da célula espermática podem ser afetadas. Por estes motivos, dentre os procedimentos utilizados nos protocolos de conservação, é comum a adição de antimicrobianos aos diluentes de sêmen, como penicilina, gentamicina, estreptomicina, tilosina, lincomicina, espectinomicina, dentre outros, visando o controle bacteriano e manutenção da qualidade espermática. No entanto, a problemática da resistência bacteriana as diversas substâncias utilizadas, bem como os efeitos deletérios de algumas delas sobre os espermatozoides, estimulam a pesquisa de novos compostos que possam substituir o uso dessas drogas ou constituírem como uma opção eficaz.

Nesse contexto, a caracterização da microbiota bacteriana reprodutiva dos catetos machos, bem como a avaliação dos efeitos da adição de substâncias antimicrobianas sintéticas e naturais para controle bacteriano durante a conservação dos espermatozoides são propostas importantes para o aprimoramento desta biotécnica, tornando-se mais uma ferramenta que poderia ser utilizada no intuito de conservação da espécie.

3 HIPÓTESES

A constituição da microbiota presente no sêmen de catetos criados em cativeiro é semelhante à dos suínos domésticos;

A carga bacteriana presente no sêmen influencia na sua qualidade;

Não se observam efeitos tóxicos de antimicrobianos sobre a função espermática *in vitro* durante o teste de termo resistência no sêmen de catetos;

Os antimicrobianos controlam o crescimento bacteriano no sêmen refrigerado de catetos, sem afetar sua qualidade espermática;

O gel da *Aloe vera* possui ação antibacteriana quando utilizada como crioprotetor na refrigeração do sêmen de catetos.

4 OBJETIVOS

4.1 Objetivo geral

Descrever a microbiota bacteriana do sêmen e prepúcio dos catetos (*Pecari tajacu*) criados em cativeiro, bem como os impactos do uso de substâncias antimicrobianas durante a conservação do sêmen dessa espécie.

4.2 Objetivos específicos

Identificar as bactérias presentes no prepúcio e sêmen dos catetos;

Quantificar e relacionar as cargas bacterianas dos isolados com os parâmetros espermáticos de qualidade no sêmen fresco;

Verificar a sensibilidade das cepas isoladas a antibacterianos utilizados em diluentes de sêmen e ao gel da *Aloe vera*;

Avaliar a toxicidade de antibacterianos adicionados ao diluente de sêmen, armazenado a 37 °C em um curto período de tempo, sobre a longevidade espermática de catetos;

Avaliar os efeitos da inclusão de antibacterianos no diluente de refrigeração de sêmen de catetos sobre a carga bacteriana e função espermática *in vitro*;

Avaliar o potencial antimicrobiano da *Aloe vera* utilizada como crioprotetor externo durante a refrigeração de sêmen de catetos.

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CAPÍTULO I

Artigo de revisão

Current and alternative trends in antibacterial agents used in mammalian semen technology

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Review Article

Running tittle: Antimicrobials in mammals' semen technology

Current and alternative trends in antibacterial agents used in mammalian semen technology

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Abstract

The use of antibacterial substances as additives in extenders for ensuring the sanitary quality of the semen employed in reproductive biotechniques and preserving it from bacterial deterioration has been reported since the mid-twentieth century. However, the deleterious effects of these drugs on the sperm quality as well as their effectiveness in controlling bacterial growth in the preserved semen have been questioned. The aim of this review was to report the antimicrobials primarily used in the extenders added to the semen of mammals, and to present alternatives to their use. Among the various mammalian species, there is a large variation regarding the antimicrobial types added to semen extenders as cephalosporins (ceftiofur, cefdinir, eg) and quinolones (ofloxacin, ciprofloxacin), alone or in combination

with large action spectra substances as penicillin-streptomycin and gentamicin-tylosin-lincomycin-spectinomycin. To combat problems related to bacterial resistance to these drugs, the emergence of alternatives is increasingly evident. Among these alternatives, use of physical methods as centrifugation and filtration, as well as the use of antimicrobial peptides and other substances from different origins have been highlighted for presenting antimicrobial potential.

Keywords: antibiotic, bacteria, chilling, cryopreservation, extenders.

Introduction

Reproduction plays an important role in ensuring the efficiency of animal production (Woelders et al., 2012), maintaining their biodiversity, and supporting the conservation programs of vulnerable or threatened species (Costa and Martins, 2008). Thus, assisted reproductive techniques such as artificial insemination (AI) associated with semen technology allow the preserved male genetic material to be used in females that are isolated from the males. This maximizes the availability of the germplasm and facilitates genetic improvement, disease control and conducting of reproductive procedures regardless of time and geographical location (Morrell and Mayer, 2017).

Although one of the benefits of AI is to reduce the spread of diseases, this technique may allow the pathogens to be easily disseminated through the transmission of contaminated semen (Foote, 2002). The semen can be contaminated particularly during the process of its collection or cryopreservation if good practices are not adopted (Zampiere et al., 2013). Moreover, the presence of pathogens can damage the sperms (Diemer et al., 2000), adversely affecting the quality of the semen subjected to cryopreservation.

For these reasons, in order to prevent bacterial multiplication, antibacterial substances are usually added to the diluents during the cryopreservation procedures. Contrarily, some of

these substances can affect the survival of sperm, the use of which can sometimes be restricted due to active regulations. Moreover, their indiscriminate use can lead to bacterial resistance, even when utilized in small quantities (Morrell and Wallgren, 2011). At this sense, this review aims to report the main antimicrobial agents added to mammalian semen extenders and the factors related to their effectiveness, as well as presenting alternatives to their use.

Sources and consequences of bacterial contamination in semen technology

Some microorganisms are present in the semen because they cross the bloodstream of the animals suffering from bacteremia. Others may come from the preputial microbiota of healthy semen donors and associate themselves with the semen during ejaculation and collection. Contamination can also occur during the processing and storage of semen, which may be derived not only from the environment but also from substances (particularly of animal origin, such as egg yolk) added to the semen extenders, equipment and materials such as storage bottles, which are in direct or indirect contact with the semen (Thibier and Guerin, 2000). Liquid nitrogen is an effective cryopreservant of pathogens and may also be an unsuspected source of contamination. This emphasizes on the need for care during the storage of semen, which should be carried out using well-protected and sealed straws (Mazurova and Krpatova, 1990).

Microorganisms can cause serious economic damages by reducing the semen quality and possible dissemination of pathogens (Prieto-Martínez et al., 2014). Contaminated semen can reduce the conception rate, cause early embryonic death and/or endometritis, clinical diseases in herds, and/or infections by unwanted pathogens (Maes et al., 2008).

It has been reported that microbes affect and interfere with the semen quality. It was verified that the presence of bacteria can cause morphological alterations, acrosome

exocytosis (Prieto-Martinez et al., 2014), sperm agglutination, decreased sperm motility and membrane integrity (Sepúlveda et al., 2014), decrease in the sperm longevity within 48 h of collection and processing (regardless of whether the diluent used is for short, medium or long term), and also causes acidification of the medium (pH between 5.7 and 6.4) (Althouse et al., 2000).

In general, it is known that the semen collection process is far from being a sterile procedure because of the involvement of multiple sources that can lead to bacterial contamination (Bussalleu and Torner, 2013). In this sense, additional measures such as regular monitoring of the animals and semen, biosafety measures to reduce contamination during collection, processing and storage, and the treatment of semen with the appropriate antimicrobials (Maes et al., 2008) are necessary.

International regulations

Several countries require the assessment of animal health for the importation of semen obtained from production animals like cattle, buffaloes, goats, sheep, horses and pigs. The regulations recommend the use of antimicrobials in semen extenders as a measure to prevent the spread of diseases and sperm degradation.

With the development of the semen trade using farm animals, regulations set by the European Union and the European Council, which required the use of antibacterial cocktails at inseminating doses, emerged in the late 1980s and early 1990s. According to the Directives 88/407 and 90/429 of the European Council, Annex C2, which lays down the health policy requirements for intra-community trade and imports of bovine and porcine semen, respectively, it is stated that an effective combination of antimicrobials, particularly against leptospires and mycoplasmas, should be added to the semen after the final dilution (EEC directive 88/407; EEC directive 90/429). The EEC directive 92/65, which applies to other

animals such as Equidae, swine and small ruminants, reports another combination of antimicrobials consisting of gentamicin (250 μg/mL), tylosin (50 μg/mL) and lincomycin/spectinomycin (150/300 μg/mL), or amikacin (75 μg/mL) and divecacin (25 μg/mL), which can be used alone (EEC directive 92/65).

Some Latin American countries also have a legislation establishing the minimum sanitary requirements for the processing and marketing of animal semen. For instance, in Brazil, laws were established only more than a decade after the implementation of the European regulations. Normative instructions for cattle (Brazil, 2003), goats and sheep (Brazil, 2014) recommended the same combination of substances used by the European Directives. However, regulations concerning the importation of swine and equine semen from the Mercosur countries neither define the substances nor the doses that may be added to the diluents.

Use of antibacterial substances in semen extenders

The antimicrobials frequently used in mammalian semen diluents include the β-lactams (penicillins, cephalosporins), which interfere with the process of bacterial cell wall synthesis, causing lysis and cell death (Spinosa et al., 2011). Others include the aminoglycosides (gentamicin, streptomycin, amikacin), macrolides (tylosin, spectinomycin) and lincosamides (lincomycin), which are inhibitors of bacterial protein synthesis (Spinosa et al., 2011). Among the various mammalian species, however, there is a large variation regarding the antimicrobial types and concentrations added to extenders used for both chilling or freezing semen procedures.

Studies on bacterial control in ruminants (Table 1) date from the 40s and 50s in the United States of America, emphasizing the importance of its use in the development of artificial insemination (AI) in bovine. Initially, the antimicrobials most used in bovine semen

technology were penicillin and streptomycin (Almquist et al., 1949). Almquist (1951) reported their use at concentrations of 1000 IU/mL and 1000 μg/mL, respectively, in an egg yolk citrate diluent and their combined use demonstrated better herd fertility results than when used alone. Two years later, Alford (1953) evidenced that diphtheroid bacilli demonstrated resistance even when high concentrations of streptomycin were added to the bovine semen. Nowadays, the use of a combination of gentamicin-tylosin-lincomycin-spectinomycin (500 μg/mL-100 μg/mL–300 μg/mL–600 μg/mL, respectively) is largely recommended for diluting the semen of several species, including bulls and buffaloes (Andrabi et al., 2001, Akhter et al., 2008, Andrabi et al., 2016). In spite of this, other antimicrobial combinations, as the ceftiofur/tylosin (200 μg/mL-100 μg/mL) and ofloxacin (100 μg/mL) have been highlighted for a more effective control of the bacterial growth in bovine semen (Gloria et al., 2014). Besides, the use of ciprofloxacin (600 μg/ml) added to the Tris-citric acid diluent has been also recommended for the preservation of buffaloes' semen (Akhter et al., 2013). On the other hand, the use of cephalosporin as cefdinir (1 mg/mL) and cefoperazone sodium (1 mg/mL) was effective for controlling bacteria during ram semen chilling at 5 °C up to 96 h (Azawi and Ismaeel, 2012). In the same species, the use of streptomycin-penicillin combination was more effective to bacterial control than lincomycin and sulfadiazinane, without causing negative effects on sperm (Moustacas et al., 2010).

Table 1 – Some antimicrobial substances successfully used in mammalian semen technology

	Antimicrobials	Concentrations	Semen diluents	Storage temperatures	Storage times	References
Bulls	Streptomycin and penicillin	100 to 1000 µg/mL or IU/mL of each	Sodium citrate-egg yolk	4,5°C	20 days	Almquist et al., 1949
	Streptomycin and penicillin (alone or combination)	1,000 mg/mL and 1,000 IU/mL	Sodium citrate-egg yolk	-	-	Almquist, 1951
	Gentamicin, tylosin, lincomycin and spectinomycin	500 µg/mL, 100 µg/mL, 300 µg/mL and 600 µg/mL	Tris-citric acid	-196 °C	1 day	Andrabi et al., 2001
	Ceftiofur and tylosin	200 µg/mL and 100 µg/mL	Bioxcell CSS I and II	-145 °C	7 days	Gloria et al., 2014
	Ofloxacin	100 µg/mL	Bioxcell CSS I and II	-145 °C	7 days	Gloria et al., 2014
Buffaloes	Gentamicin, tylosin, lincomycin and spectinomycin	500 µg/mL, 100 µg/mL, 300 µg/mL and 600 µg/mL	Skimmed milk based	5 °C	3 days	Akhter et al., 2008
	Streptomycin and penicillin G	1,000 mg/mL and 1,000 IU/mL	Skimmed milk based	5 °C	3 days	Akhter et al., 2008
	Ciprofloxacin	600 µg/mL	Tris-citric acid	-196 °C	-	Akhter et al., 2013
	Gentamicin, tylosin, lincomycin and spectinomycin	500 µg/mL, 100 µg/mL, 300 µg/mL and 600 µg/mL	Tris-citric acid	-196 °C	1 day	Andrabi et al., 2016
Rams	Streptomycin and penicillin G	1,000 mg/mL and 1,000 IU/mL	Tris-glucose	-196 °C	-	Moustacas et al., 2010
	Gentamicin	250 µg/mL	Tris-glucose	-196 °C	-	Moustacas et al., 2010
	Cefdinir	1.0 mg/mL	Sodium citrate-fructose-egg yolk	5 °C	4 days	Azawi and Ismaeel, 2012
	Cefoperazone sodium	1.0 mg/mL	Sodium citrate-fructose-egg yolk	5 °C	4 days	Azawi and Ismaeel, 2012
Boars	Gentamicin	-	-	18°C	3 days	Mazurová and Vinter, 1991
	Gentamicin and florfenicol	100 µg/mL and 100 µg/mL	Biosolwens Plus	15 °C	10 days	Bryla and Trzcinska, 2015
	Gentamicin	250 µg/mL	Beltsvile Thowing Solution	17 °C	3 days	Waberski et al., 2019
	Gentamicin	250 µg/mL	Androstar Premium	17 °C	3 days	Waberski et al., 2019
	-	-	Androstar Premium *	5 °C	3 days	Waberski et al., 2019
Stallions	Amikacin	2,500 µg/mL	Tris-egg yolk	4 °C	7 days	Arriola and Foote, 1982
	Amikacin or Ticarcillin	2,000 µg/mL	Skim milk-glucose	23 °C and 5 °C, respectively	1h and 2 days, respectively	Jasko et al., 1993
	Gentamicin or polimixin B	100 µg/mL or 1,000 IU/mL, respectively	Skim-milk glucose	20 °C and 5 °C, respectively	8h and 2 days, respectively	Vaillancourt et al., 1993
	Penicillin G and amikacin or Ticarcillin-clavulanic acid or Ceftiofur	1,000 IU/mL and 1,000 µg/mL or 1,000 µg/mL or 1,000 µg/mL	Skim-milk glucose	5 °C	1 day	Varner et al., 1998
	Gentamicin	250 µg/mL	EquiPro©	15 °C	4 days	Price et al., 2008
	-	-	EquiPro© *	5 °C	4 days	Price et al., 2008
	Cefquinome	0.99 mg/mL	EquiPro©	5 °C	2 days	Parlevliet et al., 2011
	Ticarcillin-clavulanic acid	0.5, 1.0 and 1.5 mg/mL	INRA 96® **	5 °C	3 days	Olivieri et al., 2011
	Penicillin G and amikacin	As supplied by the manufacturer	VDMZ	5 °C	3 days	Olivieri et al., 2011

	Penicillin G and gentamicin	1,000 IU/mL and 1,000 mg/mL	BotuSemen®	5 and 15 °C	1 day	Ramires Neto et al., 2015
	Penicillin G and amikacin	1,000 IU/mL and 1,000 mg/mL	TAMU	Fresh and 5 °C	1 day	Hernández-Avilés et al., 2018
	Meropenem	1,000 mg/mL	TAMU	Fresh and 5 °C	1 day	Hernández-Avilés et al., 2018
	Penicillin G and amikacin	1,000 IU/mL and 1,000 mg/mL	INRA 96® **	Fresh and 5 °C	1 day	Hernández-Avilés et al., 2019
Dogs	Gentamicin, tylosin, lincomycin and spectinomycin	250, 50, 150 and 300 µg/ml; 500, 100, 300 and 600 µg/ml; and 1000, 200, 600 and 1200 µg/ml	Tris-citric acid-fructose-egg yolk	5 °C	3 days	Becher et al., 2013
Koala (*Phascolarctos cinereus*)	Penicillin G and gentamicin	1,000 IU/mL and 100 µg/mL	PBS	16 °C	1 day	Johnston et al., 1998
Collared peccaries (*Pecari tajacu*)	Streptomycin and penicillin G	1,000 mg/mL and 1,000 IU/mL	Tris-citric acid-fructose-egg yolk	5 °C	1,5 days	Santos et al., 2019b
	Gentamicin	70 µg/mL	Tris-citric acid-fructose-egg yolk	5 °C	1,5 days	Santos et al., 2019a

*Antibiotic-free diluents; **It contains penicillin (105 µg), streptomycin (38 µg) and amphotericin B (0.315 µg) on its composition.

As early as 1968, studies had indicated that the bacteria isolated from porcine semen were also resistant to some antibacterials such as penicillin-streptomycin (Almond and Poolperm, 1996). At this sense, in the 1990s, Mazurová and Vinter (1991) reported a decrease in bacterial contamination ($<10^3$) of boar semen after dilution in a gentamicin-treated diluent incubated at 18 ° C for up to 72h. This study was pioneering at comparing gentamicin with several other antimicrobials such as ampicillin, apramycin and cefoxitin. Currently, gentamicin has established itself as one of the most used antimicrobials in boar semen diluents (Schulze et al., 2017). However, Gączarzewicz et al. (2016) demonstrated that the inhibitory activity of even gentamicin may be limited during long-term preservation (16°C for five days) in diluent (X-cell®). In fact, the storage time is an important factor related to the amount of antimicrobial in the boar semen extender. Thus, there are short-term (1 to 3 days) (Johnson et al., 1982) and long-term (more than 4 days) extenders (Haugan et al., 2007) and antibacterial concentrations are generally higher in these latter types (Table 1).

In general, the types of antimicrobials addressed in studies on stallion semen conservation are the most varied (Table 1). Arriola and Foote (1982) highlighted that bacterial strains present in equine ejaculates were resistant to common antimicrobials such as penicillin and streptomycin. Moreover, it was demonstrated that polymyxin B (Jasko et al., 1993) and gentamicin (Aurich and Spergser, 2007) could negatively affect motility parameters in cooled stallion spermatozoa, despite being potent antimicrobials in semen extenders (Vaillancourt et al., 1993). However, this information was recently contradicted by Price et al. (2008) that reported that the addition of small amounts of gentamicin (250 μg/mL) reduced bacterial growth and improved the sperm motility, velocity and viability in the stallion semen stored at 15 °C up to 96 h, compared to that of the control, which did not contain any antibacterial substance. Anyway, the cefquinome (0.99 mg/mL), a fourth-generation cephalosporin, was demonstrated as a suitable substitute for gentamicin within 48 h preservation of equine semen

at 5 ºC, providing both the bacterial control and the maintaining of sperm parameters (Parlevliet et al., 2011). Recently, Ramires Neto et al. (2015) reported that the penicillin-gentamicin combination (1,000 IU-1,000 mg/mL) in BotuSemen® (BS) (a skim milk based diluent) yielded lower bacterial load in stallion semen after cooling, compared with INRA 96®, a commercial diluent, which already contains penicillin (105 μg), streptomycin (38 μg) and amphotericin B (0.315 μg) in its composition. In fact, it was reported that the addition of clavulanic acid-associated ticarcillin (Timentin®) would be more effective for the bacterial control during equine semen preservation than the isolate use of INRA 96® diluent (Olivieri et al.,2011). In addition, the use of the potassium penicillin-amikacin combination has also been evidenced for providing efficient antibacterial action and maintaining sperm parameters during equine semen storage (Varner et al., 1998; Hernández-Avilés et al., 2018, Hernández-Avilés et al., 2019).

For companion animals, protocols for the use of antimicrobials in semen technology are generally extrapolated from other domestic animals and studies focused on the determination of effective antimicrobial concentrations are rare. For instance, Barbosa et al. (2010) found that the isolate use of penicillin at 500 IU, 1000 IU and 1500 IU / mL concentrations during canine semen cryopreservation did not control bacterial growth after thawing. Due to the worries on the *Mycoplasma* sp. and *Ureaplasma* sp dissemination through canine semen exchange, Becher et al. (2013) addressed the comparison of the effect of two antibiotic combinations, as benzylpenicillin (0.6 g/L) plus streptomycin (1.0 g/L) and the gentamicin-tylosin-lincomycin-spectinomycin (GTLS) combination at increasing concentrations (GTLS-1: 250, 50, 150 and 300 μg / ml; GTLS-2: 500, 100 , 300 and 600 μg / ml; GTLS-3: 1000, 200, 600 and 1200 μg / ml), which was demonstrated for being more effective regarding the microbial control.

Studies describing the use of antimicrobials in the preservation of semen from wild animals (Table 1) are even more scarce than in companion animals. The combination of penicillin (1000 IU/mL) and gentamicin (100 μg/mL) was effective at preserving koala semen (*Phascolarctos cinereus*) at 16 °C for 24 h as it prevented bacterial growth without interfering on the sperm motility (Johnston et al., 1998). Additionally, the addition of gentamicin (70 μg / mL) (Santos et al., 2019a) and a combination of penicillin (2000 IU/mL and 1000 IU/mL)-streptomycin (2 mg/mL and 1 mg/mL) (Santos et al., 2019b) to the semen of collared peccaries (*Pecari tajacu*) allowed the control of bacterial growth in the samples and did not present toxic effects on the quality of the chilled semen maintained up to 36 h.

Due to the differential action of antimicrobial substances in the distinct species, various studies have been conducted in order to stablish appropriate antimicrobial concentrations in the diluent. The experimental design of the studies does not follow a standardized pattern, and includes several variables, such as type and concentration of antimicrobials, preservation time, storage temperatures, previous inoculation with pathogenic bacteria and fertility trials. In this context, it is evident that the temperature is an important factor that can interfere on bacterial dissemination during semen storage, particularly for stallion and boar cooled-semen technology, in which protocols highlight use of a relatively high temperature (15 to 17 °C) in a nutrient-rich extender that can favor bacterial growth. However, it was demonstrated that even for stallions (Price et al., 2008) and boars (Waberski et al., 2019), the hypothermic storage (5 °C) may reduce the use of antimicrobial drugs (Table 1).

Alternatives to the use of antibacterial agents

The development of bacterial resistance against the main antibacterial agents used in semen extenders, such as the combination of penicillin and streptomycin (Sone et al., 1982),

amoxicillin, gentamicin, lincomycin, tylosin and spectinomycin (Althouse and Lu, 2005), has been reported. Thus, the search for alternatives that overcome bacterial resistance is a reality. Alternatives include antimicrobial peptides, physical methods for reducing bacterial load and the use of various substances, whether animal, plant or other origins.

Antimicrobial peptides

Recently, research has been focused on the use of antimicrobial peptides (AMP) that may destabilize the bacterial cell membrane (Table 2). Bussalleu et al. (2017) have investigated the use of the proline-arginine-rich antimicrobial peptide, PR-39, which belongs to the group of porcine myeloid antimicrobial peptides 36 (PMAP-36) and 37 (PMAP-37), as an additive to porcine semen extenders. The authors observed that PMAP-37 at 0.5, 1 and 3 μM concentrations reduced the bacterial load up to 10 days, besides improving the sperm viability. Moreover, the PR-39 (20 μM) promoted bacterial inhibition but it was found to be cytotoxic to the porcine sperm, whereas PMAP-36 did not exhibit any antimicrobial action.

Other peptides of porcine origin such as beta defensin-1 (PBD-1) and beta-defensin-2 (PBD-2) may be used as antimicrobial agents. Puig-Timonet et al. (2018) found that both peptides 3 mM concentration did not impair the viability and motility of the spermatozoa and were able to control microbial growth to some extent. Similarly, ε-Polylysine (40 to 128 mg/mL) was shown for effectively inhibiting bacterial growth, improving sperm quality and *in vitro* fertilization, being able to replace 50% of the gentamicin used in the extender (Shaoyong et al., 2019a).

Physical methods

Some physical methods as centrifugation and filtration (Table 2), which may reduce or replace antimicrobial use, have also been highlighted. An alternative method reported by

Morrell and Wallgren (2011) was the use of single-layer centrifugation using Androcoll™-P, a colloid based on glycidoxypropyltrimethoxysilane-coated silic, that completely removed the bacteria from 60% of the samples of swine semen and reduced the bacterial load in 40%.

Recently, the possibility of separating all sperm from seminal plasma without affecting the semen quality was investigated in swine (Morrell et al., 2019). The ejaculates were diluted in the antimicrobial-free Beltsville Thawing Solution and subjected to single-layer centrifugation in a low-density colloid, which provided an increase on sperm velocity and linearity, besides removing or reducing bacterial contamination in boar ejaculates.

In equine, Morrell et al. (2014) also evaluated the removal of bacteria by single-layer centrifugation using Androcoll™-E after the addition of bacteria (*E. coli*, *Klebsiella pneumoniae*, *Streptococcus equi* subsp. *zooepidemicus*, *Taylorella equigenitalis*, among others) in different proportions in the aliquots of semen. The reduction in counts ranged from 68% to 97% among bacteria. In another experiment involving equine semen, it was also found that colloidal centrifugation using Androcoll™-E before freezing reduced the total bacterial load after thawing and positively influenced the post-thaw motility (Guimarães et al., 2015).

Another physical method recently described for antimicrobial reduction is the seminal plasma (SP) microfiltration. The SP was separated of the sperm by centrifugation, then filtered with a 1.2 µm syringe prefilter (NalgeneTM) followed by a 0.22 µm syringe filter (NalgeneTM), and added in 20% of swine fertilization medium with and without antibiotic for AI doses. This process, in addition to reduce bacterial contamination in boar semen, has improved some parameters such as motility, plasma membrane and acrosome integrity, and mitochondrial activity (Barone et al., 2016).

Table 2 - Alternative methods for bacterial control in semen samples from mammalian species.

Alternatives	Types	Animals	References
Antimicrobial peptides	PR-39 (proline-arginine-rich antimicrobial peptide)	Boar	Bussalleu et al., 2017
	PBD-1 (beta defensin-1) and PBD-2 (beta defensin-2)	Boar	Puig-Timonet et al., 2018
	ε-Polylysine	Boar	Shaoyong et al, 2019a
Physical methods	Single-layer centrifugation (SLC)	Boar	Morrell and Wallgren, 2011, Morrell et al., 2019
	Single-layer centrifugation (SLC)	Stallion	Morrell et al., 2014, Guimarães et al., 2015
	Microfiltration	Boar	Barone et al., 2016
Miscellaneous substances	Royal jelly	Bull	Abd-Allah, 2010
		Ram	Brito et al., 2014
	Aloe vera gel	Collared peccary (*Pecari tajacu*)	Souza et al., 2016
		Bull	Farias et al., 2019
	Kojic acid	Boar	Shaoyong et al., 2019b
	Iodine methionine	Boar	Fang et al., 2017
	BactiBag®	Boar	Camugli et al., 2019
	Sodium alginate	Buffalo	Kumar et al., 2019

Other alternative substances

Some recent studies have been considering the use of new natural bioactive products (Table 2) such as phytotherapics and other compounds obtained from plants or animals to replace antibiotics in semen technology.

Abd-Allah (2010) found that the use of 0.4% royal jelly in the cryopreservation of bovine semen improved the viability and fertility characteristics of the spermatozoa. Although the study did not focus on the use of an antimicrobial activity of the jelly, it has a known antimicrobial component namely 10-hydroxy-2-decenoic acid (Blum et al., 1959), which may also have influenced the results and requires to be investigated.

Another interesting alternative would be the use of *Aloe vera*, which presents among its constituents a non-volatile fraction with bactericidal action (Radha and Laxmipriya, 2015). Its use was recently reported as an efficient cryoprotectant for ovine (Brito et al., 2014), collared peccary (Souza et al., 2016) and bovine semen (Farias et al., 2019), but its antimicrobial potential during semen preservation was not yet investigated. Besides *Aloe vera*, the *Ocimum gratissimum* leaf extract at 0.5% (Alaba and Sokunbi, 2018) as well as the essential oils of *Malaleuca alternifoli*a and *Rosmarinus officinalis*, both at 0.4 mg/mL concentration (Elmi et al., 2019), have shown satisfactory antimicrobial potential in the preservation of wild boar semen.

Other alternative to the antimicrobial drugs includes substances of various origins (Table 2) as the Kojic acid (5-hydroxy-2-hydroxymethyl-1,4-pyrone), which is a weakly acidic secondary metabolite produced by aerobic fermentation of *Aspergillus* and *Acetobacter* fungi (Song et al., 2019). It was demonstrated for inhibiting bacterial growth (at concentrations of 20 to 100 mg/mL) in diluted swine semen and for improving (40 mg/mL) sperm quality, sperm capacitation, number of sperm attached to oocyte and embryonic development (Shaoyong et al., 2019b).

Additionally, the iodine methionine, a new type of chelate amino acid, was demonstrated for inhibiting the proliferation of the phylum Proteobacteria and the genus *Staphylococcus* as well as *Pseudomonas*, and for improving sperm motility, plasma membrane integrity and acrosome integrity in swine semen after 6 days storage (Fang et al.,

2017). Recently, the IMV laboratories reported the use of the BactiBag®, a semen bag with bacteriostatic molecules, which shown potential for control bacterial growth during porcine semen storage for 3 days (Camugli et al., 2019).

Finally, the addition of sodium alginate to egg-yolk diluent improved the metal chelating capacity and antibacterial properties of the extender, besides improving antioxidant and cryoprotective activities during the cryopreservation of buffaloes semen (Kumar et al., 2019).

Final considerations

The problem of bacterial resistance has been reported since the mid-twentieth century and has stimulated the development of several studies that seek to test new antibacterial agents. However, to date, there are few well-designed studies that have aimed to evaluate the bacteriostatic/bactericidal characteristics of the antimicrobials, their effects on the semen quality of animals, and the time of bioactivity of these compounds under conditions of chilling and freezing.

In general, a variety of antibacterial substances have shown satisfactory results for ruminants and equines, in which antimicrobial drugs are used alone or in combination to increase the spectrum of action. On the other hand, the addition of gentamicin to the porcine semen diluent is well established, but in small and wild animals, studies are scarce and constitute a prominent area for further experimentation.

In parallel, the search for antimicrobial alternatives has been increasing; however, further research is needed to enable the use of adequate concentrations of these new compounds which should be effective for bacterial without impairing the sperm quality. Furthermore, in the long term, the *in vivo* effect of these substances on fertility after the use of chilled or cryopreserved semen by AI should be evaluated.

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CAPÍTULO II

Characterization of fresh semen and foreskin microbiota and its impact on sperm parameters of captive collared peccaries (*Pecari* tajacu)

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Characterization of fresh semen and foreskin microbiota and its impact on sperm parameters of captive collared peccaries (*Pecari tajacu*)

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Running title: Peccaries' reproductive microbiome

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Abstract

Aim

To evaluate the bacterial composition of collared peccary semen and foreskin, and to verify the sensitivity of isolates to antimicrobials used in semen conservation and to *Aloe vera* gel, which is an alternative external cryoprotectant.

Methods and Results

Nine foreskin and ejaculate samples from adult animals were used. Sperm characteristics and bacterial load were evaluated in fresh semen. The preputial and semen bacterial isolates were identified and tested against five concentrations of each antimicrobial (streptomycin-penicillin and gentamicin) and *A. vera* gel. *Corynebacterium* spp. and *Staphylococcus s*pp. were isolated in greater numbers than others in both semen (64.10% and 20.51%, respectively) and the foreskin (60.60% and 24.25%, respectively), and ranged from 0.4 to 21 × 10^5 colony forming units (CFU) ml^{-1}. The average load of *Corynebacterium* spp. was negatively correlated ($P < 0.05$) with the sperm membrane integrity ($r = -0.73055$) and curvilinear velocity ($r = -0.69048$). Streptomycin-penicillin and gentamicin inhibited most microorganisms, and *A. vera* showed lower antimicrobial activity.

Conclusion

Several gram-positive bacteria are present in semen and foreskin of collared peccary, and the benefits of using penicillin-streptomycin and gentamicin antimicrobials and *A. vera* in the bacterial control of diluted semen of these animals is strongly indicated.

Significance and Impact of the Study

This study provides insight into the reproductive microbiota of male captive collared peccary. This article provides a theoretical basis to assist reproductive biotechnologies for *ex situ* conservation of the species.

Keywords: bacteria; ejaculate; antibiotic; *Aloe vera*; wildlife

Introduction

Microbiota refers to a community of microorganisms that inhabit animal tissues or organs. The microbiota is host-specific and may be present in the skin (Strube *et al.* 2018), gastrointestinal tract (Oliveira *et al*. 2009; Burbach *et al*. 2017), and genitourinary tract (Mändar *et al.* 2017). Unlike that of other sites, such as the skin and intestines, little is known about genitourinary microbiota in humans (Baud *et al.* 2019), domesticated animals (Althouse and Lu, 2005; Moreno *et al.* 2016), or wild animals (Ghoneim *et al.* 2014; Johnston *et al.* 1998).

Study of the microbiome of the collared peccary (*Pecari tajacu* Linnaeus, 1758) as a representative wild species would provide information related to the composition and role of microbiome bacteria, as well as of microbe-host interactions. This knowledge would further our understanding of the nuances of the health-disease dichotomy (Human Microbiome Project Consortium, 2012). Peccaries are wild pigs that inhabit large areas of the American continent and have ecological and economic importance (Gongora *et al.* 2011). Although their global population is considered stable (Gongora *et al.* 2011), habitat loss due to destruction and fragmentation of some biomes has caused a significant decline in their population in some regions (Desbiez *et al.* 2012). The species has been successfully bred in captivity (Garcia *et al.* 2015) for scientific and economic purposes. Several studies of semen-related reproductive biotechniques have been performed (Castelo *et al.* 2010; Campos *et al.* 2014) and a recent report described the addition of *Aloe vera*, which is known to show antimicrobial activity (Cellini *et al.* 2014), to the extender (Souza *et al.* 2016).

The presence of microorganisms in semen can damage sperm (Diemer *et al.* 2000), which negatively affects the quality of semen. Issues related to bacteriospermia and infertility

can lead to controversy. On one hand, some bacteria, such as *Prevotella*, may be related to pathological conditions such as prostatitis (phylum Proteobacteria) (Mändar *et al.* 2017) and low semen quality (Baud *et al.* 2019). On the contrary, bacteria such as *Staphylococcus* and *Lactobacillus* have been related to parameters that denote good seminal quality (Mändar *et al.* 2017; Baud *et al.* 2019). Antibiotics are generally added to semen diluents to avoid the deleterious effects of bacteria (Morrell and Wallgren, 2011). Thus, knowledge of the bacterial composition and the sensitivity profile of isolated strains is valuable.

The present study evaluated the microbiology of foreskin and semen of the collared peccary, and the sperm parameters of fresh semen. In addition, we investigated the sensitivity and resistance profile of bacterial strains to the antibacterial agents commonly used in semen diluents and to *Aloe vera* gel, and determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these compounds.

Materials and methods

Ethical considerations

The procedures were performed in accordance with international animal welfare standards and submitted to the Animal Use Ethics Committee of Federal Rural University of Semiarid (UFERSA), with approval under protocol no. 23091.009851/2018-96. The experiments were approved by the Chico Mendes Institute for Biodiversity Conservation (no. 37329).

Animals

The study was conducted with animals from the Center for Wild Animals Multiplication (CEMAS) of UFERSA, which is registered with the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) as a scientific breeding facility

under approval number 1478912. CEMAS is located in Mossoró, which is a semiarid region in Brazil (5º10´S–37º10´W; average temperature range, 27–29 °C). Nine sexually mature males (mean age 40 months) were exposed to natural outdoor photoperiod (~12 h) and segregated in groups of three in paddocks (20 m × 3 m) with a covered area of 6 m^2. They were fed pig feed and fruits, with *ad libitum* access to water.

Animal restraint

The animals were fasted for 12 h. They were then caught with a dip net and anesthetized by intravenous administration of propofol (Propovan®, Cristália, Fortaleza, Brazil) in a 5 mg kg^{-1} bolus (Souza *et al.* 2009). During the procedures, a venous catheter was introduced into the cephalic vein for fluid therapy with 0.9% physiological saline solution. Vital signs were monitored.

Collection of semen and foreskin swabs

The animals were positioned in lateral decubitus, and the preputial region was cleaned using sterile saline solution. With the aid of a sterile swab, specimens were collected from foreskin surfaces, preserved in Stuart's medium, packed in isothermal boxes with recyclable ice, and sent for processing to the Veterinary Microbiology Laboratory (LAMIV) of UFERSA.

Semen was collected using an Autojac® electroejaculator (Neovet, Campinas, SP, Brazil) connected to a 12 V source. The stimulation cycle consisted of 10 stimuli at each voltage, starting at 5 V, followed by a 1 V voltage increase to 12 V. Each electrical stimulus lasted 3 s, with intermittent intervals of 2 s. The stimulation cycle lasted 10 min (Castelo *et al.* 2010). A rectal probe 15 cm long and 1.3 cm in diameter was inserted approximately 12 cm into the rectum. Semen samples were collected in sterile plastic tubes. Each sample was

divided into two aliquots. One aliquot was sent to LAMIV immediately after collection. The other aliquot was evaluated for spermatic characteristics.

Semen evaluation

After collection, semen was immediately evaluated for volume, appearance, and color. To determine sperm concentration, a 10 µl semen aliquot was diluted in 2 ml buffered formaldehyde solution (10%) and analyzed in a Neubauer counting chamber (Campos *et al.* 2014). The percentage of sperm viability was established by light microscopy (400× magnification) by counting 200 cells per slide following staining with bromophenol blue (Campos *et al.* 2014). The hypo-osmotic test was performed to assess functional integrity of the sperm cell membrane using distilled water (0 mOsm l^{-1}) as a hypo-osmotic solution (Campos *et al.* 2014). For sperm morphological analysis, semen smears were stained with Bengal Rose (Sigma-Aldrich, São Paulo, SP, Brazil) and 200 cells per slide were examined by light microscopy (1000× magnification) (Souza *et al.* 2016).

Evaluation of plasma membrane integrity was assessed using a fluorescent solution consisting of a carboxyfluorescein diacetate (CFDA) fluorophore and propidium iodide (PI) (Souza *et al.* 2016). Samples were incubated for 10 min at 37 °C with the fluorescent probes and then evaluated using fluorescence microscopy (Episcopic Fluorescent Attachment "EFA" Halogen Lamp Set; Leica, Kista, Sweden). Two hundred sperm were counted from each sample. Those that were totally green (CFDA) were intact, while those that were partially or totally red (PI) had disrupted membranes.

Sperm motility parameters were evaluated in fresh semen using an automated IVOS 7.4G system (Hamilton-Research™; Thorne, Beverly, MA, USA) using the settings previously stablished for the species (Souza *et al.* 2016). The following parameters were evaluated: total motility (%), velocity average pathway (VAP, µm/s), velocity straight line

(VSL, µm/s), velocity curvilinear (VCL, µm/s), amplitude lateral head (ALH, µm), beat cross frequency (BCF, in Hz), straightness (STR, %), and linearity (LIN, %) as well as the sperm subpopulations of rapid, medium, slow, and static.

Microbiological analysis of semen and foreskin

Semen processing began by inoculating 100 µl aliquots of each sample into 900 µl of 0.85% sterile saline (10^{-1} dilution) followed by serial dilution to 10^{-5}. Duplicate 100 µl aliquots of each dilution were plated with a Drigalski handle on the surface of Petri dishes containing Blood Base Agar (Hi Media, Mumbai, India) supplemented with 5% defibrinated sheep blood and MacConkey Agar (Hi Media, Mumbai, India) and incubated in a bacteriological incubator (Fanem LTDA, São Paulo, Brazil) at 35 ± 1 °C for 24 to 48 h. After this period, the colonies were counted and the average number of microorganisms were expressed in colony-forming units (CFU) per milliliter multiplied by the inverse of each dilution. Colonies with different aspects were isolated in Brain Heart Infusion broth (Hi Media) and identified from their macroscopic, morphotintoral, and biochemical profiles (MacFaddin, 2000).

The swabs, in turn, were washed in tubes with 2 ml of 0.85% sterile saline. An aliquot of the wash solution was inoculated with a platinum handle (2 µl) into plates containing Blood Base Agar (Hi Media) supplemented with 5% defibrinated sheep blood and MacConkey Agar (Hi Media) and incubated as described for the semen dilutions.

Obtaining A. vera gel

The *A. vera* plant was collected at the UFERSA camous, located in Mossoró/RN, which has a typical semiarid climate and annual average temperature of 27 °C. To obtain the crude extract of the plant, leaf parenchyma was extracted aseptically using sterile knives from

leaves that had been washed and dried with paper towels. The colorless gel was collected with sterile spatulas and stored in a sterile glass container until use (Souza *et al.* 2016).

Determination of MIC and MBC

The substances tested and their respective concentrations were: streptomycin-penicillin combination (0.25 mg ml^{-1} + 250 IU ml^{-1}, 0.50 mg ml^{-1} + 5000 IU ml^{-1}, 1 mg ml^{-1} + 1000 IU ml^{-1}, 1.5 mg ml^{-1} + 1500 IU ml^{-1} and 2 mg ml^{-1} + 2000 IU ml^{-1}), gentamicin (5 μg ml^{-1}, 10 μg ml^{-1}, 30 μg ml^{-1}, 50 μg ml^{-1}, and 70 μg ml^{-1}), and *A. vera* gel (2.5%, 5%, 10%, 20%, and 30%). Both antibacterial compounds and gel were diluted in Tris buffer. MIC values were determined by the microdilution method in the wells of a 96-well plate (ALAMAR®; Diadema, São Paulo, Brazil), adapted from NCCLS (2003). Bacterial strains were initially inoculated in Tryptone Soya Agar (Hi Media) and incubated at 37 °C for 24 to 48 h. The colonies were suspended in Muller-Hinton (MH) broth (Hi Media). Turbidity was adjusted to 0.5 on the McFarland scale, or to an optical density of 0.08 to 0.10 using a spectrophotometer with at a wavelength of 625 nm, equivalent to a concentration of 1×10^8 CFU ml^{-1}.

To perform the test, 100 μl of MH broth was distributed in each well of the 96-well plates. The respective concentrations of both antimicrobials or the *A. vera* gel were added (100 μl) in duplicate to the wells, so that when mixed with the broth, the ideal concentration would be reached. For use of the inoculum, the suspension adjusted to 0.5 on the MacFarland scale by diluting 1:100 in MH broth giving 1×10^6 CFU ml^{-1} as inoculum. From this suspension, 10 μl (1×10^4 CFU) was inoculated into each well already containing the substances being tested. For each inoculum, 18 wells were used: 15 were for the five concentrations of each substance tested in triplicate, and three to be used as negative control (MH broth only). A 12-well line was used for the MH broth sterility control test. The sterility of antimicrobials and *A. vera* was also tested. All tests were performed in duplicate.

After testing, the absorbance of the wells was read in an URIT-660 microplate reader (URIT Medical Electronic Co., Ltd., Guangxi, China) at a wavelength of 492 nm. Readings were obtained at time 0 (t0). The microplates were then incubated in a bacteriological incubator at 35 °C for 24 h (t24) or 48 h (t48) under aerobic conditions. The incubation was longer for slower growing microorganisms. After incubation, the absorbance values of each well were obtained in the same way.

The final average absorbance values (t24 and t48) were obtained and compared to the initial average values (t0). The MIC for each inoculum was the lowest concentration at which the final average absorbance did not differ significantly from the initial average absorbance (Leonez *et al.* 2018).

To determine the MBC, after the final absorbance reading, an aliquot of 10 µl obtained from each well was spread on the surface of MH agar and incubated at 35 °C for 48 h. The MBC was defined as the lowest concentration that resulted in no growth of the inoculated bacteria.

Statistical analyses

The results for isolated microorganisms were analyzed descriptively. Sperm characteristics and bacterial concentration were expressed as mean ± standard error (SE). The data were first examined for normality using the Shapiro–Wilk test and for homoscedasticity using Levene's test, and were transformed by log (x + 1) or arc-sine [√ (x / 100)] when necessary. For correlation analysis, scatter plots were obtained to verify the relationship between bacterial loads and sperm parameters. As we did not observe linear relationships, Spearman correlation coefficients were obtained. For the MIC, Tukey's test was used to compare the mean between the initial and final absorbance of each inoculum. Differences

were considered significant when $P \leq 0.05$. Statistical Analysis System software (SAS Inc., Cary, NC, USA) was used throughout.

Results

Fresh semen evaluation

The ejaculates of the peccaries presented with a whitish color, aqueous appearance, pH of 7.0, average volume of 3.7 ± 1.3 ml, and sperm concentration of 337.5 ± 80.9 × 10^6 sperm ml^{-1}. Table 1 shows the percentage of viable, morphologically normal sperm, with intact and functional membranes, as well as kinetic parameters of sperm motility.

Table 1 Values per individual and means (± SE) of sperm parameters in collared peccaries' (*Pecari tajacu*) fresh semen (n = 9)

General parameters	Animals									Mean ± SE
	A1	A2	A3	A4	A5	A6	A7	A8	A9	
Spermatic characteristics										
Viability (%)	85	81	88	91	89	96	84	91	69	86 ± 2.6
Normal morphology (%)	91	.	96	62	91	98	79	96	83	79 ± 4.2
Membrane integrity (%)	89	80	87	91	66	91	70	79	60	87 ± 3.8
Membrane functionality (%)	90	85	77	60	84	97	51	82	85	79.2 ± 4.9
Kinetic parameters										
Total motility (%)	82	88	87	71	92	82	48	72	66	76.4 ± 4.6
Progressive motility (%)	37	38	48	23	52	31	3	28	35	32.8 ± 4.8
Velocity curvilinear (µm s^{-1})	102.4	129.6	105.7	109.9	101.4	115.9	42.1	103.2	95.8	100.7 ± 8
Velocity straight line (µm s^{-1})	25.1	29.7	27.3	24.6	26.6	26.4	11.2	25.9	24	24.5 ± 1.8
Velocity average pathway (µm s^{-1})	49.3	63.8	49.5	48.5	49.4	46.2	16.2	48.6	42.9	46 ± 4.2
Amplitude lateral head (mm)	6.7	7.5	7.1	6.3	6.6	7.1	7.8	6.4	6.2	6.8 ± 0.2
Beat cross frequency (Hz)	30.2	27.1	32.4	35.5	28.7	39.8	41.1	33.1	31.8	33.3 ± 1.6
Straightness (%)	50	45	52	48	52	53	74	49	54	53 ± 2.9
Linearity (%)	25	23	25	23	26	22	35	24	25	25.3 ± 1.3
Subpopulations										
Rapid (%)	70	86	77	50	84	58	5	57	52	59.9 ± 8.2
Medium (%)	11	2	10	21	8	24	43	15	14	16.4 ± 4
Slow (%)	6	5	3	10	3	9	16	5	3	6.7 ± 1.4
Static (%)	13	7	10	18	5	9	35	23	31	16.8 ± 3.6

Microbiological analysis of collared peccary fresh semen and foreskin

Culture analysis revealed no sterile samples. The number of isolated colonies of each animal and the genera are described according to the source (Table 2). The number of different colonies isolated in the foreskin ranged from 2 to 5 and in the semen from 2 to 8, with 1 to 3 different genera observed in each sample.

Table 2 Bacteria identified in microbial cultivation of foreskin and fresh semen from collared peccaries (n = 9).

Animals	Foreskin		Fresh semen	
	Type of strains	Genera	Type of strains	Genera
1	2	*Corynebacterium* spp.	5	*Corynebacterium* spp. *Staphylococcus* spp.
2	3	*Corynebacterium* spp.	5	*Arcanobacterium* spp. *Corynebacterium* spp. *Staphylococcus* spp.
3	5	*Corynebacterium* spp. *Staphylococcus* spp.	4	*Arcanobacterium* spp. *Corynebacterium* spp.
4	3	*Bacillus* spp. *Corynebacterium* spp.	4	*Corynebacterium* spp.
5	4	*Corynebacterium* spp.	5	*Corynebacterium* spp. *Staphylococcus* spp.
6	3	*Corynebcterium* spp. *Rhodococcus* spp. *Staphylococcus* spp.	3	*Bacillus* spp. *Corynebacterium* spp. *Staphylococcus* spp.
7	4	*Dermabacter* spp. *Corynebacterium* spp. *Staphylococcus* spp.	2	*Bacillus* spp. *Corynebacterium* spp.
8	5	*Corynebacterium* spp. *Staphylococcus* spp.	8	*Dermabacter* spp. *Corynebacterium* spp. *Staphylococcus* spp.
9	4	*Corynebacterium* spp. *Microbcterium* spp. *Staphylococcus* spp.	3	*Dermabacter* spp. *Staphylococcus* spp.

Table 3 summarizes data of individual and mean values (± SEM) of the total bacterial load, as well as the load of each isolated microorganism in fresh semen. The average CFU ml-1 value of each isolate ranged from 0.4 to 21 × 10^5. The main bacteria isolated in both the

preputial mucosa and the ejaculate of collared peccaries were *Corynebacterium* spp. (60.60% and 64.10%, respectively) and *Staphylococcus* spp. (24.25% and 20.51%, respectively). The remaining bacteria were isolated in smaller numbers. *Arcanobacterium* sp. was identified only in semen samples (5.13%) and *Rhodococcus* spp. and *Microbacterium* spp. were isolated only in preputial samples (3.03% for each) (Table 4).

Table 3 Values per individual and means (± SE) of bacterial load (CFU ml^{-1}) in collared peccaries' (*Pecari tajacu*) fresh semen (n = 9)

Bacterial load	Animals									Mean ± SE
	A1	A2	A3	A4	A5	A6	A7	A8	A9	
Total	5×10^5	6×10^5	2.4×10^5	0.4×10^5	7.5×10^5	2.5×10^5	12×10^5	21×10^5	3.2×10^5	$8.8 \pm 2.1 \times 10^5$
Arcanobacterium spp.	-	0.4×10^5	0.4×10^5	-	-	-	-	-	-	0.4×10^5
Bacillus spp.	-	-	-	-	-	1.2×10^5	9×10^5	-	-	$5.1 \pm 3.9 \times 10^5$
Corynebacterium spp.	4.8×10^5	1.7×10^5	2×10^5	0.4×10^5	5.5×10^5	0.8×10^5	3×10^5	16×10^5	-	$4.3 \pm 1.8 \times 10^5$
Dermabacter spp.	-	-	-	-	-	-	-	4×10^5	1.8×10^5	$2.9 \pm 1.1 \times 10^5$
Staphylococcus spp.	0.2×10^5	3.9×10^5	-	-	2×10^5	0.5×10^5	-	1×10^5	1.4×10^5	$1.5 \pm 0.5 \times 10^5$

Correlation between sperm parameters and bacterial load

There was no significant correlation between the evaluated sperm parameters and the total bacterial load. However, it was verified that an increase in *Corynebacterium* sp. in collared peccary semen negatively affected ($P < 0.05$) sperm membrane integrity ($r = -0.73055$; $P = 0.0396$) as well as VCL ($r = -0.69048$; $P = 0.0480$).

Table 4 Isolates (n), relative frequency (%) and mean (± SE) load (CFU ml^{-1}) of bacteria isolated from the collared peccaries' (*Pecari tajacu*) foreskin and fresh semen (n = 9)

Genera	Foreskin		Fresh semen		
	n	%	n	%	CFU ml^{-1}
Arcanobacterium spp	-	-	2	5.13	40,000
Bacillus spp	1	3.03	2	5.13	510,000 ± 390,000
Corynebacterium spp	20	60.60	25	64.10	427,500 ± 179,172
Dermabacter spp	2	6.06	2	5.13	290,000 ± 110,000
Microbacterium spp	1	3.03	-	-	-
Rhodococcus spp	1	3.03	-	-	-
Staphylococcus spp	8	24.25	8	20.51	150,000 ± 54,650
Total	33	100.00	39	100.00	877,778 ± 250,171

Determination of MIC and MBC

Regarding the sensitivity characteristics of isolated strains, at least one colony of each bacterial genus (except *Dermabacter* spp.) was tested for MIC and MBC evaluation of two antimicrobial compounds used in mammalian semen technology (streptomycin-penicillin combination and gentamicin) and *A. vera* gel. Thirty bacteria were tested for the antimicrobials and 28 bacteria were tested for *A. vera* gel. The lowest tested streptomycin and

penicillin concentration (0.25 mg ml^{-1} + 250 IU ml^{-1}) and the 30 µg $^{-1}$ ml gentamicin concentration both inhibited 70% of the microorganisms. Higher concentrations of these drugs (2 mg ml^{-1} + 2000 IU ml^{-1} and 70 µg ml^{-1}, respectively) both inhibited 83.33% of the strains tested and were showed similar activity. Approximately 16.67% of the bacteria were resistant to the highest concentrations. These bacteria belonged to the two main isolated genera. Among them, *Corynebacterium* spp. showed greater resistance to streptomycin-penicillin, while *Staphylococcus* spp. were more resistant to gentamicin (Tables 5 and 6).

Table 5 Minimum inhibitory concentration (MIC) of streptomycin-penicillin combination against bacteria isolated (n = 30) from collared peccaries' fresh semen and foreskin

Genera	n	Concentrations (mg ml^{-1}-IU ml^{-1})				
		0.25-250	0.5-500	1.0-1000	1.5-1500	2.0-2000
Arcanobacterium spp	2	2	-	-	-	-
Bacillus spp	1	1	-	-	-	-
Corynebacterium spp	15	8	-	-	-	3
Microbacterium spp	1	-	1	-	-	-
Rhodococcus spp	1	1	-	-	-	-
Staphylococcus spp	10	9	-	-	-	-
Total	30	21	1	0	0	3
%	100.00	70.00	3.33	0.00	0.00	10.00
% inhibition of concentration		70.00	73.33	73.33	73.33	83.33

Table 6 Minimum inhibitory concentration (MIC) of gentamicin against bacteria isolated (n = 30) from collared peccaries' fresh semen and foreskin

Genera	n	Concentrations (µg ml^{-1})				
		5	10	30	50	70
Arcanobacterium spp	2	2	-	-	-	-
Bacillus spp	1	-	-	-	-	1
Corynebacterium spp	15	10	1	1	-	1
Microbacterium spp	1	1	-	-	-	-
Rhodococcus spp	1	1	-	-	-	-
Staphylococcus spp	10	4	-	1		2
Total	30	18	1	2	0	4
%	100.00	60.00	3.33	6.67	0.00	13.33
% inhibition of concentration		60.00	63.33	70.00	70.00	83.33

Testing of *A. vera* gel revealed a 21.43% inhibition of bacteria when used at the three highest concentrations tested (10%, 20%, and 30%) (Table 7). The gel presented the highest resistance (78.57%), with at least one resistant strain of each genus, except *Bacillus* spp.

Among the tested substances, streptomycin-penicillin was bactericidal only for *Bacillus* spp., *Rhodococcus* spp., and *Microbacterium* spp. Gentamicin was able to completely eliminate strains of *Arcanobacterium* spp. and *Microbacterium* spp. (Table 8).

Discussion

To the best of our knowledge, we provide novel data related to the microbiota constituents of foreskin and semen from collared peccaries. The mean value of the total semen bacterial load was similar to that found for fresh semen of swine, which is the domestic species most closely related to peccaries (Bosma *et al.* 2004), ranging from 10^3 to 10^5 CFU ml^{-1} (Schulze *et al.* 2015) and reaching concentrations up to 10^9 CFU ml^{-1} (Althouse *et al.* 2000). The deleterious effects of the presence of bacteria in animal semen are debatable.

However, some global organizations that issue guidelines on sperm parameters for the use of liquid preserved porcine semen mandate the absence of bacterial contamination, and suggest the use of antibacterial substances in semen diluents (Waberski *et al.* 2019).

Table 7 Minimum inhibitory concentration (MIC) of *Aloe vera* gel against bacteria isolated (n = 28) from collared peccaries' fresh semen and foreskin

Genera	n	Concentrations (%)				
		2,5	5	10	20	30
Arcanobacterium spp	2	-	-	1	-	-
Bacillus spp	1	-	-	1	-	-
Corynebacterium spp	13	-	1	2	-	-
Microbacterium spp	1	-	-	-	-	-
Rhodococcus spp	1	-	-	-	-	-
Staphylococcus spp	10	-	1	-	-	-
Total	28	0	2	4	0	0
%	100.00	0.00	7.14	14.29	0.00	0.00
% inhibition of concentration		0.00	7.14	21.43	21.43	21.43

In general, comparing the microbiota composition of swine and that of collared peccaries, a similarity was observed regarding the frequent isolation of *Staphylococcus*, for example, and divergence in the isolation of gram-negative bacteria, including *Proteus* spp., *Pseudomonas* spp., *Escherichia coli* (Althouse and Lu, 2005), and other Enterobacteriaceae (Okazaki *et al.* 2010). A previous study reported on the isolation of *Corynebacterium* spp. in porcine semen (Althouse and Lu, 2005), but the frequency was far less than that observed in the present study.

Table 8 Minimal bactericidal concentration (MBC) of penicillin-streptomycin combination, gentamicin and *Aloe vera* gel against bacterial isolates (n = 30) from collared peccaries' fresh semen and foreskin

Genera	n	MBC		
		Streptomycin-penicillin ($mg\ ml^{-1}$-$IU\ ml^{-1}$)	Gentamicin ($\mu g\ ml^{-1}$)	*Aloe vera* (%)
Arcanobacterium spp	2	NR	10	NR
Bacillus spp	1	250-0.25	NR	NR
Corynebacterium spp	15*	NR	NR	NR
Microbacterium spp	1	500-0.5	10	NR
Rhodococcus spp	1	250-0.25	NR	NR
Staphylococcus spp	10	NR	NR	NR

*two strains of *Corynebacterium* spp were excluded from *A. vera* gel analysis, and only 13 strains (n = 28) were tested.

NR: no results for MBC

Regarding the strains isolated from collared peccary foreskin and semen, members of the genus *Corynebacterium* are commonly found in soil, water, and the skin and mucous membranes of humans and animals (Funke *et al.* 1997). For decades, various species, including *C. renale*, *C. pilosum*, and *C. cystitidis* have been identified in the urogenital tract from several domestic mammals, such as cattle, goat, and sheep, and have been related to pyelonephritis and posthitis (Underwood *et al.* 2015). On the other hand, *Staphylococcus* spp. can be isolated from various wild species, such as Eurasian lynx (*Lynx lynx*), European otter (*Lutra lutra*), red fox (*Vulpes vulpes*), beaver (*Castor fiber*), and brown rat (*Rattus norvegicus*), and domestic animals that include cattle, calf, goat, sheep, and alpaca (Loncaric *et al.* 2019). *Staphylococcus* is an opportunistic pathogen that colonizes the upper skin, mucous membranes, and the gastrointestinal, respiratory, and urogenital tracts (Werckenthin *et al.* 2001). It is normally found in the mucous membranes of animals and is not pathogenic

under normal conditions. It is likely that collared peccaries' semen may have become contaminated during collection upon contact with the preputial mucosa. Although the pathogenicity of these microorganisms has been described in the literature, the animals used in the present study were healthy.

We did not find evidence of correlation between sperm parameters and the number of bacteria in the ejaculates of collared peccary, since the total bacterial load of fresh semen did not affect the characteristics of sperm quality. However, the presence of *Corynebacterium* spp., even for a short period after semen collection, could provoke deleterious effects on peccary sperm membrane integrity ($r = -0.73055$). Sperm damage induced by bacterial load depends on the type of bacteria, incubation period, and temperature (Bonet *et al.* 2018). Some bacteria may adhere to the sperm surface, either directly or through molecular aggregates, and either in the head (acrosomal) or intermediate area and the tail region. The adhesion can cause rupture of the sperm membrane and thickening of the structures to which they are adhered, which is related to the impairment of sperm membrane integrity and motility parameters (Bonet *et al.* 2018). We observed that the presence of *Corynebacterium* spp. also impaired VCL ($r = -0.69048$), which is a kinetic parameter of sperm motility. In equines, a significant negative correlation ($P < 0.01$) was found between total microbial load and VCL ($r = -0.664$) of semen that was diluted and stored at 5 °C only after 24 h incubation. The correlation was related to the production of reactive oxygen species (Varela *et al.* 2018). More data are needed concerning the influence of microbial load on collared peccaries' semen, and storage under refrigeration over longer periods of time is suggested to investigate interference with sperm parameters.

Sensitivity tests using the microdilution technique allowed us to phenotypically characterize the resistance of bacterial isolates in the semen of collared peccaries. We verified both MIC and MBC of the antimicrobial drugs routinely used as additives in semen extenders

for swine, namely streptomycin-penicillin (Ejaz *et al.* 2017) and gentamycin (Bryła and Trzcińska, 2015), as well as a possible alternative compound, *A. vera*, which was previously used for peccary semen technology (Souza *et al.* 2016).

Given the variety of concentrations used for antimicrobials in semen extenders (Johnston *et al.* 1998; Bryła and Trzcińska, 2015; Ejaz *et al.* 2017), we choose to test low to intermediate concentrations, focusing on the possible use of the extenders for collared peccary semen. Therefore, we avoided high doses that could be toxic to sperm and promote bacterial resistance (Morrell and Wallgren, 2011). Although bacterial resistance to streptomycin-penicillin (Sone *et al.* 1982) and gentamicin (Althouse and Lu, 2005) has been previously reported, the present study showed satisfactory results. It was found that the highest tested concentrations of both streptomycin-penicillin (2 mg ml^{-1} + 2000 IU ml^{-1}) and gentamicin (70 µg ml^{-1}) showed similar activity and promoted inhibition of > 80% of the tested microorganisms, and that low concentrations of these drugs were effective against most isolated bacterial strains. In addition, low drug concentrations had a bactericidal effect against isolated contaminating microorganisms.

Regarding *A. vera* gel, the results confirmed the antimicrobial action of the nonvolatile fraction previously described in the literature (Radha and Laxmipriya, 2015). Even though the results indicated that gel was less effective than the synthetic antimicrobials tested, it is worth noting that this gel not only acts as an alternative non-penetrating cryoprotectant for peccary sperm (Souza *et al.* 2016), but may also promote discrete control of bacterial growth in these samples. Further studies should consider testing *A. vera* gel alone or in association with other drugs for the control of bacterial load in mammalian semen.

Few studies have described genital microbiome in wild species. From what is known, some interesting factors could interfere with the microbiome. For instance, in koalas (*Phascolarctos cinereus*), *Corynebacterium* spp. were identified as the predominant

microorganism in foreskin and semen samples, regardless of whether collection involved electroejaculation or using an artificial vagina (Johnston *et al.* 1998). On the contrary, *Staphylococcus* spp. and *Bacillus* spp. were reported to be among the main isolated microorganisms in camel semen (36.71% and 19%, respectively); the bacterial load was higher in the reproductive season than in the non-reproductive period, and the bacterial load was reduced when semen was collected using an artificial vagina compared to that of electroejaculation, and after preputial lavage (Ghoneim *et al.* 2014). In this context, the characterization of the reproductive tract microbiota of captive collared peccaries is important for future studies related to reproductive biotechnologies in these animals. The microorganisms found in the semen samples of this study may have come from the animals themselves and from the environment. Since their presence in semen can cause sperm damage, proper sanitary management is necessary during the collection and handling of this material to prevent or reduce contamination. In addition, the inclusion of efficient antimicrobials in diluents may allow semen samples to be stored for long periods when destined for germplasm banks. However, further studies are needed to address the toxicity of antimicrobials to sperm cells during their preservation.

In summary, we describe the microbiome of foreskin and semen from collared peccaries. Bacterial loads ranged from 0.4 to 21 × 10^5, with emphasis on *Corynebacterium* spp. and *Staphylococcus* spp. as the main isolated mesophilic aerobic bacteria. *Corynebacterium* spp. could impair some sperm parameters. Finally, we suggest the use of streptomycin-penicillin and gentamicin as antimicrobials for the composition of peccary semen extenders, with *A. vera* gel having potential value as an alternative antimicrobial.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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CAPÍTULO III

Can antibiotics added to collared peccary (*Pecari tajacu*) semen extender affect sperm longevity during short term storage at 37 ºC?

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Will be submitted to Biopreservation and Biobanking

Qualis Capes A3

Can antibiotics added to collared peccary (*Pecari tajacu*) semen extender affect sperm longevity during short term storage at 37 ºC?

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Running title: Antimicrobial toxicity in peccaries' semen

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Abstract

The effects of antibiotics on sperm longevity in collared peccary (*Pecari tajacu*) fresh diluted semen was evaluated. Semen samples from six adult males were collected by electroejaculation and diluted in Tric-citrate-fructose alone (control) and plus streptomycin-penicillin (2 mg/ml-2000 IU/ml) or gentamicin (70 µg/ml). Membrane integrity and functionality, mitochondrial activity and sperm morphology were assessed subjectively. Sperm motility and other kinetic parameters were objectively assessed using CASA (computer-assisted semen analysis). The semen diluted according to the treatments were submitted to the thermoresistance test, incubated at 37 ° C, and the sperm parameters analyzed at 0, 30, 60, 120 and 180 min. The average values of the treatments were compared with each other and between the times. There were no differences ($P > 0.05$) between treatments until the end of the test. Control and streptomycin-penicillin samples maintained sperm function for up to 180 min (with total motility of 24.3 ± 7.1% and 28 ± 8.7%, respectively). While gentamicin aliquots retained most parameters until the end of the incubation, except for membrane integrity and mitochondrial activity that declined ($P < 0.05$) at 180 min (53.1 ± 7.1% and 50.7 ± 6.2%, respectively) compared to 0 min (80.5 ± 4.7% and 86.3 ± 3.4%, respectively). In conclusion, Tris plus streptomycin-penicillin (2 mg/ml-2000 IU/ml) or gentamicin (70 µg/ml) can be used to dilute collared peccary fresh semen incubated at 37 °C for use in reproductive biotechniques up to 180 min or 120 min, repectively.

Keywords: antibacterial, extender, sperm, thermoresistance, toxicity

Introduction

The peccary (*Pecari tajacu*) is a wild pig native to the American continent, whose global population is considered to be Least Concern (Gongorra et al., 2011). However, the

number of individuals has been reduced in some biomes as a consequence of anthropic interference through poaching and defragmentation of its habitat (Desbiez et al., 2012). At this sense, its captive breeding has been promoting the advance on the knowledge of peccary reproductive aspects, which contributes for the development of strategies for the conservation of the species (Maia et al., 2018).

Assisted reproductive techniques have made it possible to conserve the semen of these animals through chilling (Souza et al., 2016) and cryopreservation (Silva et al., 2013). Although considerable bacterial contamination of the collared peccary has already been reported (Santos et al., 2017), the use of antibiotics on the semen extender composition is not reported for the species. However, it is largely known that the presence of certain types of bacteria in semen can directly impair sperm parameters (Pinart et al., 2017) and thus interfere with fertility (Maroto Martín et al. 2010). It is suggested, therefore, the possibility of adding antibiotics as the penicillin-streptomycin combination (Toniolli et al., 2006) or the gentamicin (Waberski et al., 2019), to the semen of these animals in order to guarantee the sanitary quality of the semen doses (Morrel and Wallgreen, 2011). These drugs prevent bacterial proliferation, but they may also cause damage to sperm viability (Pinart et al., 2017; Bonet et al., 2018) according to its type and concentration (Aurich and Spergser, 2007).

The determination of the toxicity of these substances can be assessed by the semen thermoresistance test, that have already been used to assess sperm function after cryopreservation protocols in the species (Campos et al., 2014). The test will make it possible to assess the sensitivity of the sperm cell to antibiotics, thus contributing to the selection of drugs that can be added to the extender used for peccary semen preservation.

The present study aimed to evaluate the toxicity of streptomycin-penicillin and gentamicin on the sperm longevity of collared peccaries by means of the thermoresistance test in diluted semen for a short time.

Material and Methods

Animal

The Animal Use Ethics Committee of Federal Rural University of Semiarid – UFERSA (No. 23091.009851/2018-96) and the Chico Mendes Institute for Biodiversity Conservation (No. 37329) approved this study. The experiment was conducted with animals from the Center for Wild Animals Multiplication (CEMAS) of UFERSA, registered with IBAMA as a scientific breeding under number 1478912, located in Mossoró, Brazilian semi-arid region (5º10´S-37º10´W; average temperature range, 27-29ºC). Six sexually mature male (mean age 40 months) were used. The animals were exposed under natural outdoor photo period (~12 h) and segregated in groups of three in paddocks (20 m × 3 m) with covered area of 6 m^2. They were fed with pig feed and fruits, and *ad libitum* access to water.

Semen collection

The animals were fasted for 12 h prior to the semen collection procedure. They were restrained with a hand net and anesthetized through intravenous administration of propofol (Propovan®, Cristália, Fortaleza, Brazil) in bolus (5 mg/kg) (Souza et al., 2009). During the procedure, a venous catheter was introduced into the cephalic vein for fluid therapy with 0.9% physiological saline solution and the vital signs were monitored.

Semen was collected using an electroejaculator (Autojac®, Neovet, Campinas, São Paulo, Brazil), connected to a 12 V source. The stimulation cycle consisted of ten stimuli at each voltage, starting at 5 V, followed by a 1 V increase to 12 V. Each electrical stimulus lasted 3 seconds, with intermittent intervals of 2 seconds. The stimulation cycle lasted 10 minutes (Castelo et al., 2010). A rectal probe (15 cm long and 1.3 cm diameter) was inserted approximately 12 cm in the rectum.

Experimental design

The experiment was carried out in order to evaluate the existence of adverse effects of streptomycin-penicillin combination (Sigma, Sigma-Aldrich, Sao Paulo, SP, Brazil) and gentamicin (Gentatec®, Chemitec®, São Paulo, SP, Brazil) added to the Tris-citrate-fructose extender during the thermoresistance test at 37 °C of the collared peccaries fresh semen (Campos et al., 2014). The treatment groups was: control (Tris alone), Tris plus streptomycin-penicillin (2 mg/ml-2000 IU/ml) and Tris plus gentamicin (70 µg/ml). All groups were adjusted to the same sperm concentration (100 x 10^6 sperm/mL). Samples were subjected to the evaluation of the following sperm parameters: membrane integrity and functionality, mitochondrial activity, sperm morphology and kinetic parameters by means of computerized analysis. The assessments in the diluted samples were carried out at 0, 30, 60, 120 and 180 min.

Semen evaluation

After collection, semen was immediately evaluated for volume, appearance, color and pH. To determine sperm concentration, a 10 µl semen aliquot was diluted in 2 ml buffered formaldehyde solution (10%) and analyzed in a Neubauer counting chamber (Silva et al., 2014).

The hypo-osmotic test was performed to assess the functional integrity of the sperm cell membrane using distilled water (0 mOsm/l) as a hypo-osmotic solution (Santos et al., 2013). For sperm morphological analysis, semen smears were stained with Bengal Rose (Sigma-Aldrich, St. Louis, USA), being observed 200 cells/slide under light microscopy (×1000) (Sousa et al., 2013).

A semen aliquot (10µL) was incubated at 37 °C for 10 min in a solution composed of the following combination of fluorescent probes: 2 µL Propidium Iodide (PI, Sigma-Aldrich,

St. Louis, USA), 5 µl CMXRos (Mito Tracker® Red, Invitrogen®, Oregon, USA), and 3 µL Hoechst 342 (H342, Sigma-Aldrich, St. Louis, USA) (Sousa et al., 2016) Next, the samples were evaluated with an epifluorescence microscope (Episcopic Fluorescent Attachment "EFA" Halogen Lamp Set, Leica, Kista, Sweden), and 200 spermatozoa (per sample) were evaluated for the plasma membrane integrity using PI/H342 association and for mitochondrial membrane potential through CMXRos. Sperm heads marked in blue (H342) were considered to have intact membrane and those totally or partially marked with red (PI) were considered to be not intact; region of the midpiece marked in red was considered as presenting mitochondrial activity (Sousa et al., 2016)

Sperm motility parameters were evaluated by an automated IVOS 7.4G system (Hamilton-ResearchTM Thorne, Beverly, MA, USA) in fresh semen using the settings previously stablished for the species (Souza et al., 2016). The following parameters were evaluated: total motility (%), velocity average pathway (VAP, µm/s), velocity straight line (VSL, µm/s), velocity curvilinear (VCL, µm/s), amplitude lateral head (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %) as well as the sperm subpopulations: rapid, medium, slow and static.

Statistical analysis

Sperm characteristics were expressed as Mean ± SE. The data were first examined for normality by the Shapiro–Wilk test and for homoscedasticity by Levene's test and were transformed by log (x + 1) or arc-sine ($\sqrt{}$ (x/100)), when necessary. A two-way ANOVA using a general linear model using the PROC GLM procedure of the Statistical Analysis System (SAS Institute Inc.) was performed to evaluate the effects of the treatment, incubation time (0, 30, 60, 120 and 180 min) and its interaction on the studied parameters. Tukey post

hoc test was used to verify the potential differences between the means. Statistical significance was set at $P < 0.05$.

Results

The assessment of fresh semen presented a watery aspect, white color, and a volume of 1.8 ± 0.7 mL with a concentration of 503.3 ± 112.6 x 10^6 sperm/mL. Average values of 78.8 ± 3.9% motile sperm, being 86 ± 4.1% with membrane integrity, 87.2 ± 3.7% whit mitochondrial activity, 78.5 ± 3.5% morphologically normal, and 65.5 ± 6.2% with membrane functional were found in ejaculates samples.

The results related to the integrity and functionality of the plasma membrane, mitochondrial activity and sperm morphology are shown in figure 1. There were no differences ($P > 0.05$) between the treatments diluted with streptomycin-penicillin and gentamicin compared to the control (Tris alone) for any of the variables mentioned, in incubation up to 180 min. However, when assessing the progression of the variables for each treatment along the time, it was observed that the streptomycin-penicillin combination did not differ ($P > 0.05$) from the control, with both keeping the mean values stable up to 180 min (with total motility of 24.3 ± 7.1% and 28 ± 8.7% at this time, respectively), while gentamicin provoked a reduction ($P < 0.05$) at 180 min, compared to 0 min, of membrane integrity (80.5 ± 4.7% at 0 min to 53.1 ± 7.1% 180 min) and mitochondrial activity (86.3 ± 3.4% at 0 min to 50.7 ± 6.2% at 180 min) of the sperm.

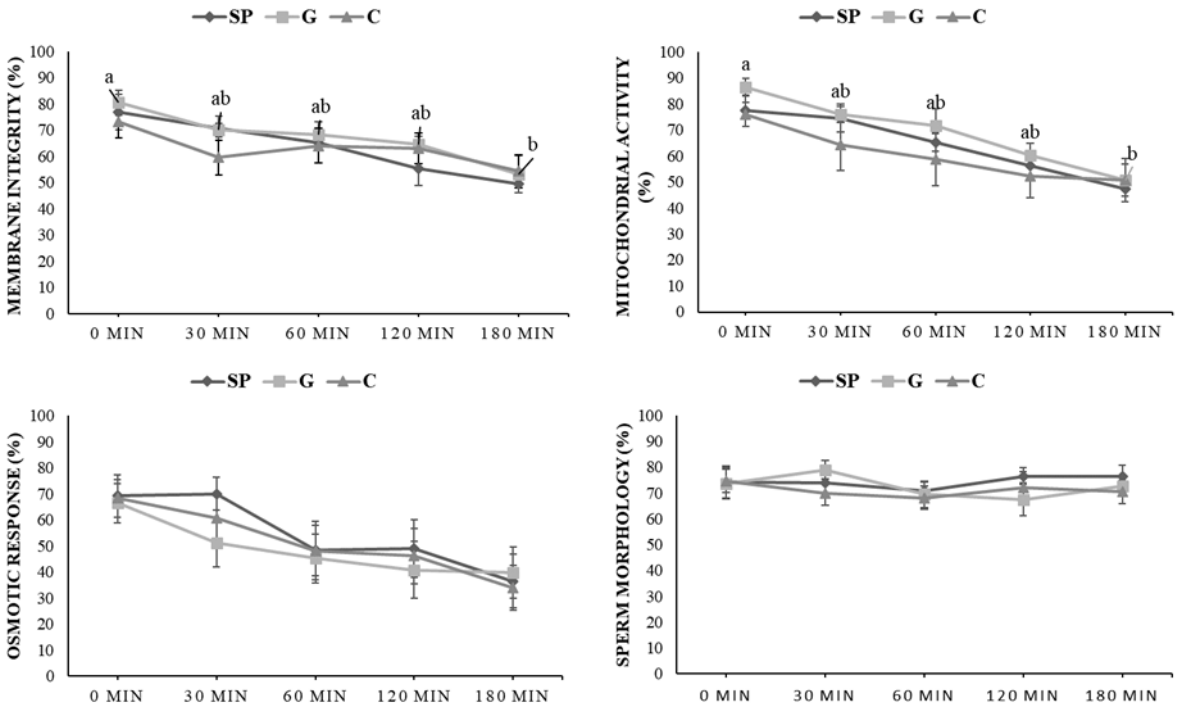

Figure 1. Values (mean) for membrane integrity (%), mitochondrial activity (%), osmotic response (%) and sperm morphology (%) on collared peccary (*Pecari tajacu*) fresh semen samples diluted in Tris-citrate-frutose without (C) and with streptomycin-penicillin (SP) or gentamicin (G) up to 180 min. (a-b) lowercase letters indicate significant differences for treatments on time ($P < 0.05$).

Regarding the analysis of motility parameters by CASA, the data are shown in table 1 and 2. No differences were observed ($P > 0.05$) among treatments or different incubation times for the kinetic variables evaluated up to 180 min.

Table 1. Values (mean ± SEM) for total (TM, %) and progressive (PM, %) motility, average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %) on collared peccary (*Pecari tajacu*) fresh semen samples diluted in Tris-citrate-frutose without (control) and with streptomycin-penicillin (SP) or gentamicin (G) up to 180 min.

		Kinetics parameters								
Treatments	**Time (min)**	**TM**	**PM**	**VAP**	**VSL**	**VCL**	**ALH**	**BCF**	**STR**	**LIN**
Tris-control	0	66.3 ± 9.7	31.3 ± 8.6	38.6 ± 3	23.5 ± 2	80.4 ± 7.3	5.9 ± 0.3	29.2 ± 1.6	59.8 ± 2.2	30.3 ± 1.3
	30	48.8 ± 12.8	21.3 ± 6.6	44.8 ± 4.5	26.1 ± 2.5	86.5 ± 8.1	6 ± 0.3	28.7 ± 1.6	53.3 ± 1	29.2 ± 1.4
	60	47.3 ±7.2	17.1 ± 4.7	39.2 ± 2.3	20 ± 1.2	78.3 ± 5.7	5.6 ± 0.2	29.2 ± 1.6	49.7 ± 1.1	26 ± 0.4
	120	37 ± 9.7	13.8 ± 3.9	37.7 ± 3.8	20.5 ± 2.4	75.5 ± 5.6	5.6 ± 0.3	26.9 ± 1.3	52.3 ± 3.3	26 ± 1.5
	180	24.3 ± 7.1	5.1 ± 3	32.9 ± 5.8	14.2 ± 1.3	66.7 ± 12	4.9 ± 0.5	28.2 ± 1.5	48.5 ± 2.1	26.1 ± 2.1
Tris-SP	0	57.2 ± 12.2	30 ± 10.4	39.7 ± 3.6	24.4 ± 2.4	86 ± 7.9	6 ± 0.3	30.4 ± 1.6	58.7 ± 3.5	28.2 ± 1.2
	30	53.8 ± 13	26.2 ± 10.3	40 ± 5.4	23 ± 3.4	85.8 ± 10.3	6.3 ± 0.4	30.8 ± 2	55.8 ± 2.1	27 ± 0.7
	60	50.8 ± 14.4	25.5 ± 8.8	37.2 ± 5.6	23 ± 2.3	82.3 ± 11.2	6 ± 0.4	25.9 ± 2.6	57.3 ± 1.2	29 ± 2
	120	56.7 ± 10.4	25 ± 7.2	34.2 ± 3.1	19.6 ± 1.8	78.3 ± 6	5.9 ± 0.3	29.4 ± 2	55.2 ± 1.3	25.2 ± 0.6
	180	28 ± 8.7	8.7 ± 4.6	32 ± 2.8	17.7 ± 1.6	72.1 ± 4	5.2 ± 0.4	28.1 ± 1.7	52.5 ± 1.1	24.8 ± 1.3
Tris-G	0	52.5 ± 10.9	25.7 ± 10	38.4 ± 2.7	22.9 ± 2.1	82.8 ± 6.9	5.8 ± 0.2	29.5 ± 1.2	57.8 ± 3.3	28 ± 1.5
	30	52 ± 15.2	29.1 ± 10.2	41.4 ± 4.1	24.8 ± 2	87.8 ± 8.5	5.8 ± 0.3	26.3 ± 0.9	59.5 ± 2.4	29.2 ± 0.7
	60	47.3 ± 13.6	19.5 ± 6.8	37.1 ± 2.6	21.3 ± 1.6	81.9 ± 8.5	5.9 ± 0.2	28.5 ± 2.5	56.1 ± 3.1	25.8 ± 1.7
	120	21 ± 5.5	4.5 ± 1.5	30.3 ± 2.8	15.2 ± 0.5	68.9 ± 4.8	5.2 ± 0.3	29.4 ± 2.4	50 ± 3.4	22.8 ± 1.7
	180	18.5 ± 3.1	2 ± 0.6	24.5 ± 2.1	13.3 ± 1.3	57.2 ± 3.5	4.5 ± 0.5	33.2 ± 2.4	55.3 ± 2.5	26 ± 1.6

No differences were found between treatments and storage times ($P > 0.05$).

Discussion

The formation of biobanks containing valuable germplasm is important for wildlife conservation programs. One of the tools related to the biobank and assist in the maintenance of endangered species is the conservation of sperm (Barbas and Mascarenhas, 2009; Comizzoli, 2017). In this sense, the present study aggregates results that collaborate with the use of collared peccary semen for application in Assisted Reproductive Technologies (ART) (Andrabi and Maxwell, 2007), assisting in the use of antibacterial additives to the collared peccary semen extender.

Table 2. Values (mean ± SEM) for motile subpopulations on collared peccary (*Pecari tajacu*) fresh semen samples diluted in Tris-citrate-frutose without (control) and with streptomycin-penicillin (SP) or gentamicin (G) up to 180 min.

Treatments	Time (min)	Motile subpopulations			
		Rapid (%)	Medium (%)	Slow (%)	Static (%)
Tris-control	0	44.8 ± 6.6	0.5 ± 4.7	0.2 ± 1.1	28.7 ± 9.9
	30	36.5 ± 10.3	0.3 ± 3.6	0.3 ± 0.9	44.5 ± 12.6
	60	33.8 ± 6.7	0.4 ± 3	0.3 ± 0.9	44.8 ± 6.8
	120	28 ± 8.6	0.3 ± 3	0.2 ± 1.2	57.3 ± 10.8
	180	11.8 ± 6.2	0.3 ± 4.1	0.2 ± 0.6	70.8 ± 7.6
Tris-SP	0	40.3 ± 4.4	0.4 ± 4.4	0.2 ± 1	38 ± 12
	30	39.5 ± 3.2	0.4 ± 3.2	0.2 ± 2	40.2 ± 12.8
	60	37.7 ± 3.4	0.3 ± 3.4	0.2 ± 0.9	44.8 ± 14.8
	120	37.8 ± 2.7	0.4 ± 2.7	0.2 ± 1.1	37.3 ± 10.2
	180	16.5 ± 3.2	0.3 ± 3.2	0.2 ± 0.7	67.5 ± 8.8
Tris-G	0	35.8 ± 3.5	0.4 ± 3.5	0.2±0.9	42.3±10.8
	30	38.8 ± 4.5	0.3 ± 4.5	0.2±1.6	44±15.1
	60	31.8 ± 5	0.4 ± 5	0.2 ± 1.5	46.7 ± 14
	120	11.1 ± 3.2	0.3 ± 3.2	0.2 ± 1.1	75.5 ± 6.2
	180	5.5 ± 2.3	0.4 ± 2.3	0.2 ± 0.7	76.2 ± 3.4

No differences were found between treatments and storage times ($P > 0.05$).

The results showed that the streptomycin-penicillin combination at a concentration of 2 mg/mL-2000 IU/mL did not cause adverse effects on the structure and function of the sperm cell in the 37 °C resistance test. In an pervious experiment on the sensitivity of bacterial strains isolated from semen of collared peccaries, it was found that concentrations of streptomycin-penicillin ranging from 0.5 mg/ml - 500 IU/ml to 2 mg/ml - 2000 IU/ml inhibited from 70% to over 80% of the isolates, respectively. This higher concentration used is twice the most used in semen extenders of domestic species such as bull (Almquist, 1951), dogs (Lopes et al., 2009), ram (Moustacas et al., 2010) and buffaloes (Ahkter et al., 2008). However, there is a variation in doses for different species, from 38 µg/mL-105 µg/mL (accompanied by 0.315 µg/ml amphotericin) in stallions (Dean et al., 2012) to 10 mg/mL-10,000 IU/mL in wild canids (*Canis lupus* and *C. lupus baileyi*) (Zindl et al., 2006)

It is added that the inclusion of streptomycin-penicillin and gentamicin alone in the diluents is important in the control or elimination of certain pathogens that may be present in the semen (Moustacas et al., 2010), for example. Being able to guarantee the sanitary quality of the semen doses by controlling possible contamination during its handling. Streptomycin and gentamicin is aminoglycoside antibiotics that block the production of protein by binding to the 30S ribosome, thus inhibiting messenger RNA in the bacterial cell (Luzzatto et al., 1968; Hahn and Sarre, 1969), and penicillin is a β-lactams antibiotic which interferes bacterial cell wall synthesis, causing lysis, and cell death (Waxman and Strominger, 1983).

In turn, gentamicin at a concentration of 70 µg/mL caused effects on the integrity of the plasma membrane and on the mitrochondrial activity after 120 min of incubation at 37 °C. Membrane integrity is essential for sperm function and survival, and the loss of integrity could decrease fertilization capacity and lead to cell death (Rodriguez-Martinez and Barth, 2007), and the mitochondria produce the ATP, which is an essential source of energy for sperm motility, (Silva and Gadella, 2006). Despite these informations, this antibiotic is

generally used in higher doses such as 250 mg/mL in swine semen extenders (Waberski et al, 2019). being used mainly during the refrigeration of the semen of these animals (Bryła and Trzcińska, 2015). Thus, it is also suggested that the toxicity test of these substances be implemented in the conservation of chilled or cryopreserved semen, in order to evaluate the effect of antibacterials on sperm cells submitted to adverse conditions such as cold.

Despite the possible deleterious effects caused by gentamicin, some authors report that this drug can cause mitochondrial dysfunction and overproduction of reactive oxygen species (ROS) in mammalian cells, which lead to oxidative damage to DNA, proteins and membrane lipids (Kalghatgi et al., 2013). Mitochondria share ribosomes and protein synthesis machinery similar to bacteria (Elliott and Jiang, 2019). It is also known that gentamicin kills bacteria by binding to its 30S ribosomal unit and, thus, affecting protein synthesis (Hahn and Sarre, 1969). Thus, it is suggested that the mitochondria of eukaryotic cells, similar to evolutionary bacteria, would be affected by antibiotics in the same way as bacteria (Elliott and Jiang, 2019), suggesting possible toxic mechanism in sperm. Among other effects caused by gentamicin in mammalian cell culture, we can highlight the upregulates of expression gene of hypoxia inducer factor 1 alpha (HIF1a), glycolytic enzymes and glucose transports in mammalian cell culture, besides increase the lactate production of the cell lines (Elliott and Jiang, 2019).

The present study used the thermoresistance test as a screening for the selection of antibiotics that can be used during the collared peccary semen chilling (Souza et al., 2016) and cryopreservation (Castelo et al.,2010). Since the semen storage temperature can influence bacterial control (Waberski et al., 2019), it is necessary to test antibiotics at these different temperatures. The way of assessing sperm viability by the thermoresistance test is widespread. Test was also carried out on the species, but to assess the influence of different diluents on the quality of semen (Campos et al., 2014) and epididymal sperm (Bezerra et al.,

2014) after freeze-thaw. In other wild species, the effect of centrifugation and diluents on the viability of giant armadillo sperm (*Euphractus sexcintus*) (Sousa et al., 2014) and on the longevity of epididymal sperm in cavy (*Galea spixii*) was evaluated (Silva et al., 2017), both studies being carried out within 180 min. Thermoresistance tests at 37 °C after semen collection have already been also performed in order to assess the influence of seminal diluents on sperm viability in dogs (Moura et al., 2002; Uchoa et al., 2002).

In conclusion, the collared peccary semen diluted in Tris plus penicillin-streptomycin or gentamicin could be maintained at 37 °C for up to 180 min or 120 min, respectively, after collection without toxic effects on sperm functions. In this context, the present study contributes to the understanding of the types of antibiotics that can be used in semen conservation techniques, as well as for the time limitations on the transport of uncooled collared peccary semen between geographic areas separated by a short distance.

Author Disclosure Statement

No conflicting financial interests exist.

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CAPÍTULO IV

Effects of antibacterial drugs and *Aloe vera* on bacterial growth and sperm function during chilled semen storage in collared peccary (*Pecari tajacu*)

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ABSTRACT

Toward developing strategies for the conservation of collared peccaries, this study evaluated the effect of antibiotics and *Aloe vera* on the bacterial load and sperm quality of peccary semen following liquid storage. Two concentrations of streptomycin-penicillin (SP1: 1 mg/mL - 1000 IU/mL and SP2: 2 mg/mL - 2000 IU/mL) and gentamicin (G3: 30 µg/mL and G7: 70 µg/mL) were added to the extenders, Tris-egg yolk (TE) and Tris-*Aloe vera* (TA), during the chilling of semen at 5°C. Bacterial growth and sperm characteristics, including membrane integrity and function, mitochondrial activity, morphology and kinetic patterns of motility, were evaluated for 36 h. The treatments containing SP2, SP1, and G7, controlled ($P < 0.05$) bacterial growth (ranging from $0.5 \pm 0.3 \times 10^3$ to $10 \pm 4.1 \times 10^3$ colony forming unit (CFU)/mL) for 36 h of semen storage regardless of the diluent. Treatments diluted in TE plus antibiotics did not show difference ($P > 0.05$) in the bacterial load or sperm quality. TA treatments, with or without antibiotic, affected ($P < 0.05$) membrane integrity and mitochondrial activity after 12 h. The TE-G7 treatment maintained the sperm parameters for longer, including the total (41.9 ± 6.1%) and progressive motility (15 ± 2.6%) at 24 h, as well as membrane integrity (58.3 ± 2.1%) and curvilinear velocity (76.7 ± 5.8%) at 36 h. In conclusion, we recommend the use of the TE extender along with gentamicin (70 µg/mL) or alternatively streptomycin-penicillin (1 mg/mL - 1000 IU/mL) for the liquid storage of collared peccary semen.

Keywords: Antimicrobials; Microorganisms; Biobanking; Wildlife

1. Introduction

The collared peccary (*Pecari tajacu*) is a wild pig which is widely distributed across the American continent. It inhabits forests, shrublands, savannas, deserts, and grasslands from

the south of United States of America to the north of Argentina (Gongora et al., 2011). Collared peccaries have important ecological roles as seed dispersers and serve as prey for large carnivores (Desbiez and Keuroghlian, 2009). They are also of economic importance for their meat and skin (Nogueira-Filho et al., 2004). Although their population has been relatively stable worldwide (Gongora et al., 2011), it has been declining in some biomes in the recent years as a consequence of anthropic actions that destroy the habitats and fragments the areas occupied by these animals (Desbiez et al., 2012).

Due to their growing demand and commercial production potential, the collared peccaries have been successfully bred in captivity (Garcia et al., 2015). This may have a scientific purpose for the conservation and sustainable use of the species, and also an economic purpose, related to the animal's productive aspects. For these reasons, the species has become the target of several studies in assisted reproductive techniques, with the purpose of improving semen conservation protocols, including cryopreservation (Castelo et al., 2010; Silva et al., 2013; Campos et al., 2014) or chilling (Souza et al., 2016), in which *Aloe vera* gel has been successfully tested as an alternative cryoprotectant.

There are, however, some health risks associated with these semen conservation techniques, including the potential to transmit pathogens and infectious diseases (Eaglesome and Garcia, 1997) through artificial insemination. Bacteria can contaminate the semen during collection and processing if good sanitation practices are not adopted (Zampiere et al., 2013). It has been reported that the presence of certain types of bacteria in swine semen can directly impair sperm parameters and thus interfere with fertility (Maroto-Martín et al. 2010; Úbeda et al., 2013; Pinart et al., 2017). For these reasons, antibiotics are usually added to semen extenders to prevent bacterial growth and multiplication. However, the indiscriminate use of antibiotics can lead to the development of bacterial resistance, even when low doses of antibiotics are used (Morrell and Wallgren, 2011).

The antibiotics that are most commonly used in the composition of extenders for semen preservation in various species, including bovines (Almquist, 1951) and equines, (Dean et al., 2012) include a combination of streptomycin and penicillin. Gentamicin has been established as the main antimicrobial for use in semen extenders in swines (Schulze et al., 2017), the domestic species most closely related to the peccaries (Bosma et al., 2004). Despite the routine use of these drugs, the search for new antimicrobial alternatives continues (Morrell and Wallgren, 2014). *Aloe vera* gel has been suggested as a viable option due to its antibacterial properties (Kumar et al., 2019).

In this context, the present study aimed to determine the effect of the addition of streptomycin-penicillin and gentamycin in an extender with or without *Aloe vera* gel on liquid preservation of collared peccary semen by evaluating the bacterial load and sperm characteristics.

2. Materials and methods

2.1. Ethical considerations

All experimental procedures were performed in accordance with the international animal welfare standards and approved by the Animal Use Ethics Committee of the Federal Rural University of Semi-arid – UFERSA (No. 23091.009851/2018-96). All the experiments were approved by the Chico Mendes Institute for Biodiversity Conservation (No. 37329).

2.2. Animals and semen collection

The study was conducted with animals from the Center for Wild Animals Multiplication (CEMAS) of UFERSA, which is located in Mossoró, a Brazilian semi-arid region (5º10´S-37º10´W; average temperature range, 27 - 29ºC) and registered as a scientific breeding center (IBAMA No. 1478912). Ten sexually mature males (mean age 40 months)

were used for the study. The animals were exposed to natural outdoor photo period (~12 h) and segregated into groups of five in paddocks (20 m × 3 m) with covered area of 6 m^2. They were fed with pig feed and fruits, and provided *ad libitum* access to water.

The animals were fasted for 12 h prior to the semen collection procedure. They were restrained with a hand net and anesthetized through intravenous administration of propofol (Propovan®, Cristália, Fortaleza, Brazil) in bolus (5 mg/kg) (Souza et al., 2009). During the procedure, a venous catheter was introduced into the cephalic vein for fluid therapy with 0.9% physiological saline solution and the vital signs were monitored.

Semen was collected using an electroejaculator (Autojac®, Neovet, Campinas, São Paulo, Brazil), connected to a 12 V source. The stimulation cycle consisted of ten stimuli at each voltage, starting at 5 V, followed by a 1 V increase to 12 V. Each electrical stimulus lasted 3 seconds, with intermittent intervals of 2 seconds. The stimulation cycle lasted 10 minutes (Castelo et al., 2010). A rectal probe (15 cm long and 1.3 cm diameter) was inserted approximately 12 cm in the rectum. Semen samples were collected in sterile plastic tubes and divided into two aliquots. One of the aliquots was immediately evaluated for bacterial load, and the other was subjected to sperm analysis.

2.3. Experimental design

The experimental protocol was designed to compare two concentrations of the streptomycin-penicillin combination (Sigma, Sigma-Aldrich, São Paulo, Brazil) and gentamicin (Gentatec®, Chemitec®, São Paulo, Brazil) added to the Tris-citrate-fructose extender (Castelo et al., 2010) supplemented with either of two external cryoprotectants, egg yolk and *Aloe vera* gel, during the refrigeration (5°C) of the collared peccary semen. The composition of the treatment groups is shown in Table 1. All groups were adjusted to the same sperm concentration (100 × 10^6 sperm/mL). Samples of fresh and diluted semen from

each animal were subjected to quantification of aerobic mesophilic bacteria and evaluated for the following sperm characteristics: membrane integrity and functionality, mitochondrial activity, sperm morphology, and kinetic metrics using computerized analysis. The assessments of the samples were carried out following dilution (0 h) every 12 h for up to 36 h.

Table 1. Composition of the extenders on the experimental groups.

Extenders	Antibiotics	Groups
Tris-egg yolk (TE)	None (control)	TE-C
	Streptomicym-penicillin (2 mg/mL-2000 IU/mL)	TE-SP2
	Streptomicym-penicillin (1 mg/mL-1000 IU/mL)	TE-SP1
	Gentamicin (70 μg/mL)	TE-G7
	Gentamicin (30 μg/mL)	TE-G3
Tris-*Aloe vera* (TA)	None (control)	TA-C
	Streptomicym-penicillin (2 mg/mL-2000 IU/mL)	TA-SP2
	Streptomicym-penicillin (1 mg/mL-1000 IU/mL)	TA-SP1
	Gentamicin (70 μg/mL)	TA-G7
	Gentamicin (30 μg/mL)	TA-G3

2.4. Obtaining Aloe vera gel

The *Aloe vera* plant was collected from the campus of the UFERSA, located in Mossoró (Rio Grande do Norte, Brazil) which has a typical semi-arid climate and annual average temperature of 27°C. To obtain the crude extract (a colorless gel) of the plant, the parenchyma of its leaves was extracted aseptically. Briefly, the leaves were washed, dried with paper towels, cut with sterile knives and the gel was collected using sterile spatulas. The gel was then stored in a sterile glass container until use (Souza et al., 2016).

2.5. Bacterial quantification

Semen processing began with the inoculation of 100 µl aliquots of each sample into 900 µl of 0.85% sterile saline (dilution 10^{-1}) followed by serial dilution to 10^{-5}. Then, 100 µl aliquots of each dilution were plated with a Drigalski handle on the surface of Petri dishes containing Plate Count Agar (Hi Media, Mumbai, India). All samples were tested in triplicates and plates were incubated in a bacteriological incubator (Fanem LTDA, São Paulo, Brazil) at 35 ± 1°C for 24 - 48 h. Following incubation, the colonies were counted and the average number of bacteria was expressed as CFU per milliliter multiplied by the inverse of each dilution (Tortora et al., 2017).

2.6. Semen analysis

The semen was evaluated for volume, appearance, color, and pH immediately after collection. To determine the sperm concentration, a 10 µl aliquot of the semen was diluted in 2 ml of buffered formaldehyde solution (10%) and analyzed using a Neubauer counting chamber (Silva et al., 2014).

The hypo-osmotic test was performed to assess the functional integrity of the sperm cell membrane using distilled water (0 mOsm/L) as a hypo-osmotic solution (Santos et al., 2013). For morphological analysis of the sperm, semen smears were stained with Bengal Rose (Sigma-Aldrich, St. Louis, USA) and observed under a light microscope (×1000; 200 cells/slide) (Sousa et al., 2013).

A semen aliquot (10 µL) was incubated at 37 °C for 10 min in a solution composed of the following combination of fluorescent probes: 2 µL Propidium Iodide (PI, Sigma-Aldrich, St. Louis, USA), 5 µL CMXRos (Mito Tracker® Red, Invitrogen®, Oregon, USA), and 3 µL Hoechst 342 (H342, Sigma-Aldrich, St. Louis, USA) (Sousa et al., 2016). Next, the samples were evaluated with an epifluorescence microscope (Episcopic Fluorescent Attachment "EFA" Halogen Lamp Set, Leica, Kista, Sweden). A total of 200 spermatozoa (per sample)

were evaluated for the plasma membrane integrity using PI/H342 association and for mitochondrial membrane potential through CMXRos fluorescence. The sperm heads marked in blue (H342) were considered to possess intact membranes and those totally or partially marked in red (PI) were considered to be not intact; sperms with regions of the midpiece marked in red were considered as presenting mitochondrial activity (Sousa et al., 2016).

Sperm motility parameters were evaluated using an automated IVOS 7.4G system (Hamilton-Research^{TM} Thorne, Beverly, MA, USA) using the settings previously established for the species (Souza et a., 2016). The following parameters were evaluated: total motility (%), velocity average pathway (VAP, µm/s), velocity straight line (VSL, µm/s), velocity curvilinear (VCL, µm/s), amplitude lateral head (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %) as well as the sperm subpopulations: rapid, medium, slow, and static.

2.7. Statistical analysis

Sperm characteristics and bacterial concentration are expressed as mean ± Standard Error (SE). The data were first examined for normality using the Shapiro - Wilk test and for homoscedasticity using Levene's test and were transformed by log (x + 1) or arc-sine ($\sqrt{}$ (x/100)), when necessary. A two-way ANOVA using a general linear model using the PROC GLM procedure of the Statistical Analysis System (SAS Institute Inc.) was performed to evaluate the effects of the treatment, incubation time (0, 12, 24, and 36 h) and its interaction on the studied parameters. Tukey post-hoc test was used to verify the potential differences between the means. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Fresh semen evaluation

The ejaculates of the peccaries presented a whitish color and aqueous appearance, with a pH of 7.4 ± 0.2. The average volume was 1.9 ± 0.4 ml with sperm concentration of 128.6 ± 40.7 × 10^6 sperm/ml. The average value for total motile sperm was 79.4 ± 3.0%, with 83.1 ± 1.6% and 72.2 ± 3.4% presenting with intact membranes and functional membranes, respectively. In addition, an average of 77.7 ± 2.8% of sperms presented with normal morphology and 82.8 ± 1.3% with mitochondrial activity. A bacterial load of 2.3 ± 0.9 × 10^6 CFU/mL was found in fresh semen samples.

3.2 Bacterial load assessment

Bacterial growth was observed in 70.25% (281/400) of the diluted semen samples and the bacterial loads observed during semen storage are shown in Figure 1. Regardless of the extenders used, only samples containing streptomycin-penicillin combination at either of the two concentrations (2 mg/mL - 2000 IU/mL – SP2; 1 mg/mL - 1000 IU/mL – SP1) controlled 100% of the bacterial growth for 36 h in the samples in 40% and 30% of the animals, respectively. Further, the results of bacterial growth control demonstrated that SP1 and SP2 and the higher gentamicin (70 µg/mL) concentration (G7) controlled the bacterial load during the entire storage period of 36 h. The results for the group treated with the *Aloe vera* isolate (group Tris-*Aloe vera* control – TA-C) were similar ($P > 0.05$) to that of the group treated with egg yolk (group Tris-egg yolk control – TE-C) throughout the storage period and both failed to control bacterial growth in the samples.

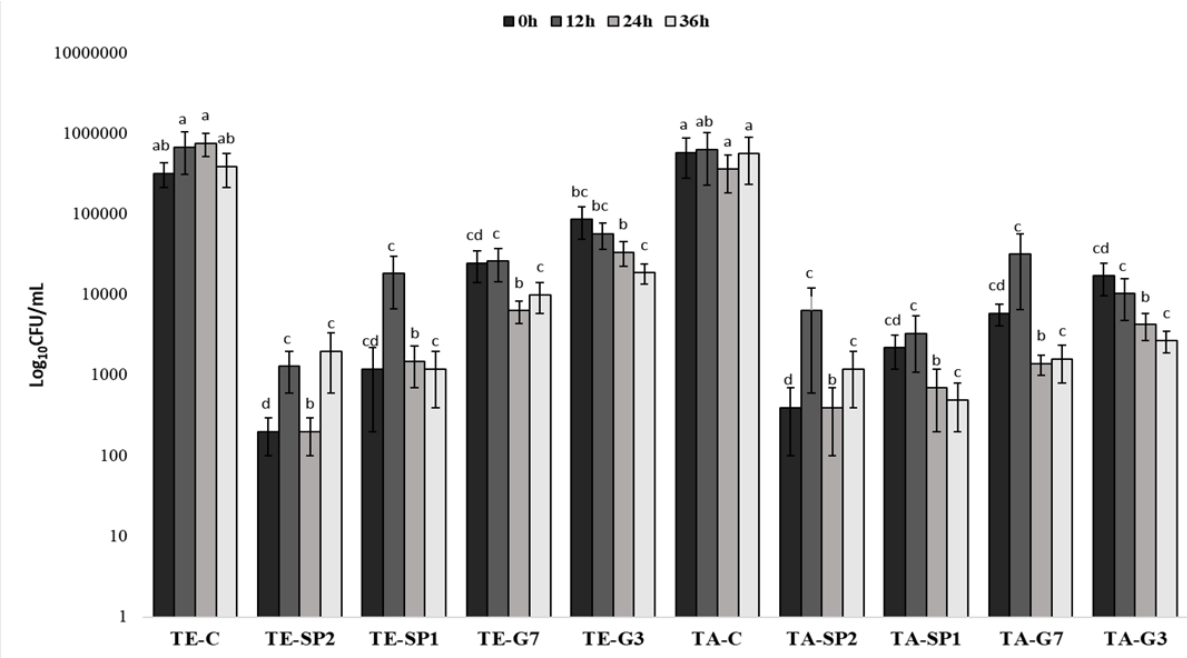

Fig. 1. Values (mean ± SEM) for bacterial load (Log_{10} CFU/mL) in collared peccaries (*Pecari tajacu*) chilling (5 °C) semen (n=10) diluted in Tris-egg yolk (TE) and Tris-*Aloe vera* (TA) with and without different antibiotics concentrations (control – C, 2 mg/mL-2000 IU/mL of streptomycin-penicillin – SP2, 1 mg/mL-1000 IU/mL of streptomycin-penicillin – SP1, 70 µg/mL of gentamicin – G7 and 30 µg/mL of gentamicin – G3) storage up to 36h. (a-d) lowercase letters indicate significant differences for treatments at the same time ($P < 0.05$). No differences were found between storage times.

3.3. Chilled semen analysis

The use of any SP combination or gentamicin at any concentration added to the TE extender provided more efficient preservation ($P < 0.05$) of the sperm membrane integrity and mitochondrial activity compared to all the TA groups with or without antibiotics (Table 2). It is also noteworthy that the analyzed parameters were stable for longer in samples extended in TE, both in the control and plus antibiotics groups. During the preservation for 36 h, the sperm membrane integrity was most effectively preserved in the groups TE-C, TE-SP1, TE-G7 and TE-G3 (TE plus gentamicin 30 µg/mL). The mitochondrial potential did not vary over

time in the TE-C and TE-G3 treatments, while in the other treatments (TE-SP1, TE-SP2, and TE-G7) there was a decrease in the mitochondrial potential ($P < 0.05$) at 36 h or earlier. As regard to the preservation of membrane functionality and sperm morphology (Table 2), there were no significant differences among treatments or storage times during the semen preservation for 36 h.

Evaluation of the sperm motility and kinetic parameters (Table 3) revealed that treatments diluted in TE, especially TE-C, TE-SP1, and TE-G7, provided more efficient ($P < 0.05$) preservation of total and progressive sperm motility compared to the treatments containing TA at 12 and 24 h. Despite the progression over time, there was a significant decrease ($P < 0.05$) in total motility over time (compared to 0 h) in all groups, with samples diluted in TA showing decreased total motility earlier (12 h) compared to those diluted in TE. Even in the groups containing TE, samples diluted in TE-SP2 showed a decrease in total motility values earlier (24 h) compared to the other treatments (TE-C, TE-SP1, TE-G7 and TE-G3) in which it declined only at 36 h. Further, only TE-G7 maintained ($P < 0.05$) progressive motility for long periods (only declining at 36 h), demonstrating that it was the most effective treatment for semen preservation.

Among the other sperm kinetic parameters obtained using CASA (Table 3), both the extenders, TE and TA, with or without antibiotics provided adequate preservation values for average pathway velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL) during the storage for 36 h. When the treatments were compared with each other, all the samples diluted in TE provided higher average values for VAP ($P < 0.05$) than those diluted in TA, regardless of the antibiotics used. An important finding regarding the progression of velocities over time was that only two treatments, TE-SP2 and TE-G7, maintained ($P > 0.05$) the VCL values during storage for 36 h.

Table 2. Values (mean ± SEM) for plasma membrane integrity. mitochondrial activity osmotic response and sperm morphology in collared peccaries (*Pecari tajacu*) chilling (5 °C) semen (n=10) diluted in Tris-egg yolk and Tris-*Aloe vera* with and without different antibiotics concentrations.

Parameters	Time	Groups									
		Tris-egg yolk					Tris-Aloe vera				
		Control	SP2	SP1	G7	G3	Control	SP2	SP1	G7	G3
Sperm membrane integrity (%)	0h	71.9 ± 2	79.1 ± 3.4^{A}	79.8 ± 2.5	78.1 ± 2	73.8 ± 2	61 ± 3.5^{A}	65.3 ± 5.1^{A}	66.9 ± 4^{A}	62.5 ± 2.7^{A}	56.8 ± 3.6^{A}
	12h	64.5 ± 5.4^{ab}	68.7 ± 4.9^{aA}	66.4 ± 3.1^{a}	70.1 ± 3.4^{a}	66.2 ± 3.5^{a}	40.4 ± 4.3^{cAB}	34.7 ± 5.9^{cB}	41.5 ± 7.1^{bcB}	39.5 ± 5.4^{cAB}	39.3 ± 8^{cAB}
	24h	60.9 ± 3.5^{a}	56.4 ± 4.8^{aA}	62.2 ± 3.4^{a}	67.5 ± 3.3^{a}	60.5 ± 3.2^{a}	29 ± 6.6^{bB}	27.8 ± 5^{bB}	30.6 ± 3.5^{bB}	22.6 ± 4.8^{bB}	28.5 ± 5.6^{bB}
	36h	56.6 ± 3.5^{a}	51.1 ± 4.2^{aB}	59.3 ± 2.9^{a}	58.3 ± 2.1^{a}	60 ± 2.6^{a}	22.4 ± 4^{bB}	23.8 ± 4.4^{bB}	24.3 ± 4.6^{bB}	24.5 ± 3.8^{bB}	20.7 ± 5.1^{bB}
Mitochondrial activity (%)	0h	71 ± 3.2	75.9 ± 2.6^{A}	80.5 ± 2.8^{A}	78.5 ± 1.9^{A}	72.3 ± 1.7	60.5 ± 4.4^{A}	74.1 ± 3.4^{A}	63.9 ± 6.6^{A}	66.4 ± 4.5^{A}	63.7 ± 4.3^{A}
	12h	63.3 ± 3.1^{a}	64.7 ± 4.3^{aAB}	63.5 ± 2.7^{aAB}	69 ± 3.2^{aAB}	60.8 ± 3.9^{a}	37.6 ± 3.3^{bAB}	31.1 ± 5.4^{bB}	34.1 ± 7.7^{bB}	33.2 ± 5.8^{bB}	32.4 ± 6^{bB}
	24h	56.5 ± 3.1^{a}	49.1 ± 4.8^{aB}	58.7 ± 3^{aAB}	67 ± 3.3^{aAB}	58.9 ± 3.4^{a}	25 ± 6^{bB}	23.6 ± 3.8^{bB}	21.9 ± 3.4^{bB}	23.1 ± 5.6^{bB}	20.8 ± 3.1^{bB}
	36h	53.1 ± 2.6^{a}	49.8 ± 5.3^{aB}	57.3 ± 3.5^{aB}	53.6 ± 4.2^{aB}	58.1 ± 3.5^{a}	17.3 ± 3.6^{bB}	20.9 ± 4.5^{bB}	22.2 ± 5.1^{bB}	17.6 ± 3.6^{bB}	16.9 ± 4.3^{bB}
Osmotic response (%)	0h	55 ± 5.9	64.9 ± 3.3	60.9 ± 3.4	50.3 ± 4.4	50.6 ± 5.3	52.1 ± 4.9	57 ± 3.7	59.6 ± 5	56.1 ± 3.4	57.3 ± 6.1
	12h	51.3 ± 5.4	60 ± 4.5	56.4 ± 4.1	61.7 ± 3.6	54.8 ± 4.7	48.7 ± 5.9	44.8 ± 4.7	38.2 ± 4.8	45 ± 5.7	42.1 ± 6.4
	24h	56.4 ± 6.5	59 ± 4.3	60.4 ± 5.4	57.1 ± 5.9	61.4 ± 5.9	39 ± 7.1	44.6 ± 6.7	37.1 ± 6.7	37.8 ± 5.8	34.3 ± 6.7
	36h	43.8 ± 6.6	50 ± 6.2	47.5 ± 4.4	43.9 ± 4.9	50.7 ± 4.7	31.9 ± 3.1	41.1 ± 8.1	31.9 ± 5.6	37.7 ± 4.9	35.7 ± 5.4
Normal morphology (%)	0h	74.5 ± 3.8	73.6 ± 3	73.8 ± 3,5	75.2 ± 3,6	75.3 ± 3,9	74.8 ± 3,2	71.5 ± 3,2	72.6 ± 3,7	75.5 ± 3,2	73.4 ± 3,3
	12h	71.2 ± 4	71.1 ± 3,6	73.3 ± 3,5	72.5 ± 3,6	72.5 ± 3,4	72.5 ± 3,8	72.7 ± 3,6	71.4 ± 3,4	72.2 ± 3,6	71.1 ± 3,6
	24h	67.8 ± 3,4	71.2 ± 4,5	71.7 ± 3,9	68.8 ± 4	68.8 ± 4,5	71.2 ± 4,5	72.3 ± 3,3	69.8 ± 4,3	70.8 ± 4,2	67.9 ± 4,2
	36h	68.3 ± 4.8	68.7 ± 4,2	66.1 ± 4,1	70.1 ± 4,5	71.4 ± 5	68.8 ± 3,9	70.8 ± 4,2	69.6 ± 5,3	67.5 ± 4,6	67.8 ± 4,8

[a-c] Values are expressed as mean ± SEM with different letters in rows differ significantly ($P < 0.05$).

[A-B] Values with different letters in columns differ significantly ($P < 0.05$).

In addition, it was found that in treatments containing *Aloe vera*, the percentage of sperm with rapid motility decreased while the number of static sperm increased at 12 h, which was different from the treatment with egg yolk (that varied at longer storage periods). Among these, TE-G7 treatment resulted in maintenance of the number of fast sperm for up to 24 h, while TE-SP1 treatment conserved the subpopulation of static sperm which did not increase during the entire storage period. Both TE-G7 and TE-SP1 treatments were similar to the TE control for the two variables.

Further, data obtained using CASA including amplitude of lateral head (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), and medium, and slow motility sperm, showed no relevant differences. They are detailed in the supplementary material.

4. Discussion

To our best knowledge, this is the first study to analyze the effect of antibiotics on collared peccary semen conservation. As demonstrated in the study, the inclusion of a combination of streptomycin-penicillin or gentamicin, especially to the Tris extender containing egg yolk, provided efficient control of bacterial load, while also preserving peccary sperm parameters for 36 h under chilling storage.

The positive effect of using gentamicin at the higher concentration (70 µg/mL) was evidenced by the effective bacterial growth control during peccary semen chilling. This drug is one of the most commonly used antimicrobials in swine semen extenders (Schulze et al, 2017), and provides effective bacterial control during liquid storage of semen (Waberski et al., 2019). However, development of bacterial resistance to this drug has been reported recently (Gączarzewicz et al., 2016). Like all aminoglycoside antibiotics, gentamicin blocks the production of protein by binding to the 30S ribosome, thus inhibiting messenger RNA in the bacterial cell (Hahn and Sarre, 1969).

Table 3. Values (mean ± SEM) for sperm motility kinetic parameters in collared peccaries (*Pecari tajacu*) chilling (5 °C) semen (n=10) diluted in Tris-egg yolk and Tris-*Aloe vera* with and without different antibiotics concentrations.

		Groups									
		Tris-egg yolk					Tris-Aloe vera				
Parameters	**Time**	**Control**	**SP2**	**SP1**	**G7**	**G3**	**Control**	**SP2**	**SP1**	**G7**	**G3**
Total motility (%)	0h	66.1 ± 4.4 ^{A}	50.3 ± 5.4 ^{A}	61.8 ± 4.3 ^{A}	63.5 ± 5.5 ^{A}	63.7 ± 5 ^{A}	50.2 ± 9.1 ^{A}	61.8 ± 4.3 ^{A}	66.9 ± 6.6 ^{A}	58.7 ± 7.1 ^{A}	58.2 ± 7.9 ^{A}
	12h	49.9 ± 6.9^{aAB}	28 ± 6.4^{abAB}	42.8 ± 6.2^{aAB}	47.2 ± 5.7^{aAB}	35.7 ± 5.7^{abAB}	9.6 ± 4.2^{cB}	13.3 ± 4^{bcB}	12.4 ± 4.9^{bcB}	11.7 ± 3.3^{bcB}	12.6 ± 2.7^{bcB}
	24h	42.8 ± 7.3^{aAB}	15.3 ± 4.4^{acB}	34.7 ± 6.2^{aAB}	41.9 ± 6.1^{aAB}	34.1 ± 8.2^{abAB}	8.7 ± 2.9^{bcB}	5.4 ± 1.2^{cB}	7.7 ± 2.3^{bcB}	6.8 ± 1.5^{bcB}	4.9 ± 1.4^{cB}
	36h	30.7 ± 6.2^{aB}	9.1 ± 2.4^{abB}	25.6 ± 5.9^{abB}	20.3 ±5.5^{abB}	26.3 ± 4.8^{abB}	7.8 ± 3^{bB}	6 ± 2.7^{bB}	4.4 ± 0.8^{bB}	6.8 ± 3.7^{bB}	16.9 ± 4.3^{bB}
Progressive motility (%)	0h	36.8 ± 4.9 ^{A}	23.1 ± 4.7 ^{A}	32.7 ± 6.7 ^{A}	35.9 ± 5.8 ^{A}	37.4 ± 5.9 ^{A}	17 ± 4.7 ^{A}	23.9 ± 6.7 ^{A}	25.2 ± 7.8 ^{A}	24.4 ± 5.4 ^{A}	22.8 ± 6.9 ^{A}
	12h	15.6 ± 1.9^{aAB}	6.5 ± 1.6^{abAB}	14.2 ± 2.2^{aAB}	18.1 ± 2.6^{aAB}	9.6 ± 1.3^{abB}	1 ± 0.4^{bB}	2.5 ± 1^{bB}	2.3 ± 1.2^{bB}	2.1 ± 0.9^{bB}	2.3 ± 0.4^{bB}
	24h	13.5 ± 2.5^{aB}	3.1 ± 1^{abB}	10.7 ± 2.1^{abB}	15 ± 2.6^{aAB}	10.6 ± 3.1^{abB}	1.7 ± 0.7^{bB}	1 ± 0.3^{bB}	1.1 ± 0.5^{bB}	1.5 ± 0.4^{bB}	1.2 ± 0.4^{bB}
	36h	11.8 ± 2.9^{aB}	2.2 ± 0.9^{abB}	6.4 ± 2.2^{abB}	7.2 ± 2.1^{abB}	9.2 ± 1.7^{abB}	1 ± 0.4^{bB}	1.1 ± 0.4^{abB}	1 ± 0.4^{abB}	1.7 ± 1^{abB}	0.5 ± 0.2^{bB}
Average path velocity (µm/s)	0h	51.4 ± 3.4	38.5 ± 0.9	45.6 ± 2	46.2 ± 1.8	50.3 ± 2.5	38 ± 3.5	38.9 ± 1.9	40.4 ± 2	42.7 ± 2.5^{A}	39.3 ± 2.2^{A}
	12h	44.4 ± 1.7^{ab}	36 ± 3.3^{ac}	44.3 ± 2.8^{ab}	48 ± 3.6^{a}	44.8 ± 2.3^{ab}	28.5 ± 2.4^{c}	31.2 ±3.4b^{c}	28.4 ± 1.7^{c}	31 ± 1.5^{bcAB}	32.6 ± 1.8^{bcAB}
	24h	45.7 ± 2.7^{ab}	32.2 ± 2.1^{bcde}	40.3 ± 2.2^{abcd}	47.8 ± 1.9^{a}	41.4 ± 3.8^{abc}	31.9 ± 1.5^{bcde}	30.5 ± 1.7^{cde}	26.2 ± 3.5^{de}	30.3 ± 2.5^{cdeAB}	24.1 ± 4.3^{eB}
	36h	39.8 ± 2.7^{ac}	28.9 ± 4.1^{ac}	33.8 ± 1.5^{ac}	40 ± 3.4^{ab}	41.5 ± 2.1^{a}	25.4 ±1.8^{c}	28.8 ± 2.7^{ac}	27.2 ± 3.7^{ac}	25.8 ± 1.6^{bcB}	25.9 ± 1.7^{bcB}
Straight line velocity (µm/s)	0h	30.6 ± 1.5	23.7 ± 0.9	29.5 ± 2^{A}	28 ± 1.7	30.1 ± 1.3	21.1 ± 1.4	21.8 ± 1.4	22.4 ± 1.8	22.9 ± 1.2	21.4 ± 1.8
	12h	23.6 ± 1.4^{ab}	19.9 ± 2.2^{ab}	24 ± 1.9^{abAB}	27.3 ± 2.6^{a}	23.3 ± 1.7^{ab}	14.2 ± 1.4^{b}	14.8 ± 1.4^{b}	16.6 ± 1.1^{b}	17.2 ± 1.3^{ab}	17.6 ± 1^{ab}
	24h	25 ± 1.6^{ab}	17.4 ± 1.6^{ac}	21.8 ± 1.6^{acAB}	26.6 ± 1.3^{a}	21.8 ± 2.6^{ac}	18.1 ± 1.4^{ac}	19 ± 2.6^{ac}	16.1 ± 2.3^{bc}	17.3 ± 2.6^{ac}	13.5 ± 3.2^{c}
	36h	22.7 ± 2.1^{a}	16.8 ± 2.6^{ab}	17.8 ± 1.5^{abB}	22.9 ± 2.2^{a}	23.3 ± 1.4^{a}	12.3 ± 1.1^{b}	19.6 ± 2.5^{ab}	14.7 ± 1.3^{ab}	15 ± 0.9^{ab}	15.9 ± 2.1^{ab}
Curvilinear velocity (µm/s)	0h	114.2 ± 5.9^{aA}	82.9 ± 1.5^{c}	104 ± 6.8^{acA}	104.1 ± 3.9^{ac}	112.7 ± 4.6^{abA}	82.6 ± 6.9^{cA}	85.2 ± 4.3^{bcA}	86.1 ± 6.1^{acA}	93.3 ± 4^{acA}	86.2 ± 4.5^{acA}
	12h	89.1 ± 3.5^{abAB}	66.7 ± 6^{ac}	84.6 ± 6.2^{acAB}	93.8 ± 6^{a}	88.9 ± 6.1^{abAB}	56.5 ± 3.5^{cAB}	62.2 ± 6.1^{bcAB}	56.1 ± 4.5^{cB}	57.7 ± 3.6^{cB}	64.1 ± 4.4^{bcAB}
	24h	89.4 ± 5.3^{abAB}	61.2 ± 3.7^{bc}	76.1 ± 4.3^{acB}	93.4 ± 4^{a}	79.2 ± 8.2^{abB}	65.6 ± 3.5^{acAB}	55.5 ± 2.3^{bcB}	49.4 ± 6.5^{cB}	57.8 ± 4.4^{bcB}	49.7 ± 8.7^{cB}
	36h	78.9 ± 4.7^{abB}	61.7 ± 4.9^{ac}	66 ± 3.6^{acB}	76.7 ± 5.8^{ab}	80.6 ± 3.8^{aB}	51 ± 3.7^{bcB}	54.4 ± 4.6^{acB}	53.1 ± 7.2^{acB}	53.1 ± 5.8^{acB}	47.6 ±2.2^{cB}
Rapid motility (%)	0h	52.8 ± 4.7 ^{A}	33.4 ± 5 ^{A}	47.7 ± 5.9 ^{A}	50.1 ± 6.3 ^{A}	52.7 ± 6.6 ^{A}	31.5 ± 6.3 ^{A}	37.3 ± 7.6 ^{A}	40.5 ± 9.8 ^{A}	41.3 ± 6.5 ^{A}	35.3 ± 8.2 ^{A}
	12h	33.1 ± 4.2^{aAB}	14.3 ± 3.7^{acAB}	29.4 ± 4.9^{abAB}	34.8 ± 4.6^{aAB}	23.9 ± 4.1^{acB}	2.3 ± 0.8^{cB}	6.3 ± 2.4^{bcB}	5 ± 2.5^{bcB}	5.1 ± 1.8^{bcB}	5.6 ± 0.9^{bcB}
	24h	29.6 ± 5.6^{aAB}	7.6 ± 2.9^{abB}	22.8 ± 5.1^{abB}	30 ± 4.9^{aAB}	23.2 ± 6^{abB}	3.8 ± 1.2^{bB}	2.1 ± 0.6^{bB}	2.6 ± 1^{bB}	2.7 ± 0.7^{bB}	2.3 ± 0.7^{bB}
	36h	22.3 ± 4.9^{B}	4.7 ± 1.7^{B}	14.8 ± 3.8^{B}	14.8 ± 4.4^{B}	18.6 ± 3.9^{B}	2.2 ± 0.8^{B}	2.2 ± 0.7^{B}	1.7 ± 0.6^{B}	3.1 ± 1.8^{B}	1.2 ± 0.4^{B}
Static (%)	0h	30.1 ± 4.2	44.5 ± 6 ^{A}	34.3 ± 4.6	33.4 ± 5.6 ^{A}	32.7 ± 4.9 ^{A}	45.3 ± 9.3 ^{A}	39 ± 7.5 ^{A}	39.9 ± 10.3 ^{A}	37.4 ± 7.3 ^{A}	42.6 ± 9.7 ^{A}
	12h	42.6 ± 7.1^{b}	65.8 ± 6.7^{abAB}	50.1 ± 6.3^{b}	45.8 ± 6.2^{bAB}	57.8 ± 6.1^{abAB}	87 ± 5.7^{aB}	83.7 ± 4.3^{aB}	85 ± 5.6^{aB}	85.3 ± 3.6^{aB}	84.3 ± 2.7^{aB}
	24h	50.7 ± 7.9^{d}	80.4 ± 5.3^{adB}	59.1 ± 6.9^{cd}	51.2 ± 6.8^{dAB}	61.4 ± 8.9^{bdAB}	89.2 ± 3^{abcB}	92.6 ± 1.3^{abB}	90.3 ± 2.6^{abB}	91 ± 2^{abB}	93.6 ± 1.8^{aB}
	36h	62.6 ± 6.8^{b}	87.7 ± 3^{abB}	68.6 ± 6.6^{ab}	75.2 ± 6.6^{abB}	68.9 ± 5.4^{abB}	89.3 ± 3.9^{abB}	91 ± 3.3^{abB}	93.9 ± 0.7^{aB}	91.2 ± 4.2^{aB}	94.9 ± 1.6^{aB}

$^{a-e}$ Values with different lowercase letters in rows differ significantly ($P < 0.05$).

$^{A-B}$ Values with different uppercase letters in columns differ significantly between times of the same parameter ($P < 0.05$).

We also highlight the effectiveness of streptomycin-penicillin combination at both the concentrations tested (SP2 and SP1), which not only controlled bacterial growth, but also eliminated the microorganisms in some peccary semen samples stored under chilling conditions. This combination has been added to semen extenders since the 1950s in various species including bovine (Almquist, 1951), ovine (Moustacas et al., 2010), and equine (Dean et al., 2012), despite reports of resistance that date decades ago (Alford, 1953). Its use in refrigerated semen is reported at concentrations that vary between species and range from 38 µg/mL - 105 µg/mL (in combination with 0.315 µg/ml amphotericin) in stallions (Dean et al., 2012) to 1 mg/mL - 1000 IU/mL in dogs (Lopes et al., 2009), which is the concentration used currently for the majority of domestic species. The effectiveness of the drug combination is related to their synergistic mechanism of action, which provides a broad spectrum of action and bactericidal potential. Streptomycin is an aminoglycoside antibiotic with gentamicin-like action (Luzzatto et al., 1968), and penicillin is a β-lactams antibiotic which interferes bacterial cell wall synthesis, causing lysis, and cell death (Waxman and Strominger, 1983).

During peccary semen preservation, no bacterial control was noted with the extender containing only *Aloe vera* gel. Moreover, no synergistic antibacterial effect among the gel and the antibiotics was evidenced. On the contrary, the bacterial load of the extender containing *Aloe vera* gel was similar to the bacterial load of the extender containing egg yolk, despite its reported antimicrobial potential (Kumar et al., 2019). Among the *A. vera* metabolites associated with antibacterial activity, the roles of anthraquinones and the glucomannan and acemannan polysaccharides have been highlighted (Maan et al., 2018). However, secondary metabolites contents can be influenced by several factors such as seasonality, rainfall, radiation, temperature, level of nutrients and water, age of the plant, among others (Gobbo-Neto and Lopes, 2007), which are inherent and vary according to the place of study.

Variability in *Aloe* vera gel metabolites could also be a reason for the lower effectiveness of the extender containing the gel for preserving peccary sperm membrane integrity and mitochondrial activity when compared to the extenders containing egg yolk. Once mitochondria produce ATP, which is an essential source of energy for sperm motility (Silva and Gadella, 2006), the decrease in mitochondrial activity could also impair the kinetic motility parameters following 12 h storage. In fact, the study that proposed the use of *Aloe vera* as an alternative external cryoprotectant for peccary sperm also evidenced a decrease in sperm motility parameters at 36 h under liquid storage at 5ºC (Souza et al., 2016). In addition, recent studies have demonstrated that *Aloe vera* when used as a cryoprotectant (5%, 10%, and 20%) also affected the motility of chilled bovine sperm (Farias et al., 2019), as well as the motility and membrane functionality of swine sperm (10%, 20%, and 30%) (Nunes et al., 2019). The differences in the results between studies stems from the inherent differences in the sperms of different species and from the lack of standardization of *Aloe vera* gel, highlighting the needs for the chemical characterization of *Aloe vera* gel (Farias et al., 2019).

In the present study, the most efficient preservation of sperm motility parameters and membrane integrity was obtained when peccary semen was chilled in the extender containing egg yolk, supplemented with the higher concentration of gentamicin (G7, 70 µg/mL). Gentamicin has reportedly been used as an additive in porcine semen extenders (Schulze et al., 2017) although at higher doses such as 200 µg/mL (Bryła and Trzcińska, 2015) and 250 µg/mL (Waberski et al., 2019). In porcines, gentamicin did not interfere with sperm motility parameters during long-term semen storage (6 days at 15°C) and when combined with other drugs such as florfenicol and polimixin B, it improved progressive motility and mitochondrial potential (Bryła and Trzcińska, 2015). It is necessary to highlight the importance of establishing an appropriate gentamicin concentration for semen extender for different species as reported in this study for collared peccaries. The significance of using an optimum dose

was illustrated in a report on stallions, in which the use of 1 mg/mL gentamicin adversely affected sperm viability and motility during chilling, and also impaired the VAP, VSL, and VCL (Aurich and Spergser, 2007).

Collared peccary sperm also showed sensitivity to streptomycin-penicillin at high concentrations (2 mg/mL - 2000 IU/mL, SP2), which provided reduced values for sperm membrane integrity, mitochondrial activity, and other motility parameters, even when used in extenders supplemented with egg yolk. One possible explanation for this is that bactericidal antibiotics (quinolones, aminoglycosides, and β-lactams) cause mitochondrial dysfunction and overproduction of reactive oxygen species (ROS) in mammalian cells, which leads to oxidative damage to DNA, proteins, and membrane lipids (Kalghatgi et al., 2013). Similar results were observed in bulls, where the use of 4000 IU/mL penicillin affected sperm motility, while streptomycin, at doses of up to 8 mg/mL had no effect on this parameter (Sykes and Mixner, 1951). In fact, the toxic effect of the drugs may be related to the variable sensitivity of sperms from different species, since a 10 × higher than standard concentration (10 mg/mL - 10,000 IU/mL) of SP was used in the extender for cryopreservation of semen from wild canids (*Canis lupu*s and *C. lupus baileyi*) (Zindl et al., 2006).

At general, the bacterial load did not appear to affect the quality of the diluted peccary semen, which showed optimal results even in the control groups. However, in addition to the bacterial load, the deleterious effects on sperms also depend on the type of bacteria, incubation period, and temperature (Pinart et al, 2017; Bonet et al, 2018). Another important observation from the present study was that the average bacterial load in the peccary semen diluted in TE and TA remained lower than that in fresh semen. This demonstrated an optimal control of contamination of the semen resulting from the use of aseptic techniques and sterile materials during semen collection and manipulation in the experiments.

This study complements the efforts of other research groups in the area of conservation of collared peccary semen and contributes an improved protocol for peccary semen storage by identifying antibiotics suitable for use in the chilling extenders. This improved protocol for peccary semen storage may be safely used in assisted reproductive technologies (ART) such as in vitro fertilization (IVF) or artificial insemination (AI).

5. Conclusion

In conclusion, gentamicin (70 µg/mL) or streptomycin-penicillin combination (1 mg/mL - 1000 IU/mL), as an alternative, may be effectively used as additives in TE extenders during storage of collared peccary semen at 5 ºC, to control bacterial growth and maintain sperm longevity for 36 h.

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Author contribution statement

C.S. Santos, A.M. Silva, L.B. Campos, E.C.G. Praxedes, S.S.J. Moreira, M.C.G. Rebouças, J.B.F. Souza Júnior, N.D. Alves, F.M.C. Feijó, M.F. Oliveira and A.R. Silva performed the experiment and collected the data generated. All authors contributed to analysis, interpretation of the result and writing of the paper.

Competing interest statement

None of the authors have any conflict of interest to declare.

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Suplementary Table – The results of the assessment of other collared peccaries' (*Pecari tajacu*) sperm kinetic parameters (n=10) in chilling (5 °C) semen diluted in Tris-egg yolk and Tris-*Aloe vera* with and without different antibiotics concentrations.

		Groups									
		Tris-egg yolk					**Tris-Aloe vera**				
Parameters	**Time**	**Control**	**SP2**	**SP1**	**G7**	**G3**	**Control**	**SP2**	**SP1**	**G7**	**G3**
Amplitude of lateral head (µm)	0h	6.8 ± 0.2	6.4 ± 0.3	6.9 ± 0.3	6.4 ± 0.2	6.9 ± 0.2	5.7 ± 0.2	5.9 ± 0.2	5.7 ± 0.4	6.3 ± 0.2^{A}	5.4 ± 0.6
	12h	6.5 ± 0.3	5.8 ± 0.4	6.2 ± 0.2	6.4 ± 0.2	6.6 ± 0.2	3.9 ± 0.7	4.6 ± 0.8	4.5 ± 0.5	4.8 ± 0.7^{AB}	5.3 ± 0.5
	24h	6.7 ± 0.2^{a}	5.4 ± 0.5^{ab}	6.2 ± 0.3^{ab}	6.7 ± 0.3^{a}	5.4 ± 0.7^{ab}	4.4 ± 0.6^{ab}	5.4 ± 0.7^{ab}	5.3 ± 0.6^{ab}	4.1 ± 0.7^{abAB}	3.6 ± 0.9^{b}
	36h	6.1 ± 0.3^{ab}	4.2 ± 0.8^{ac}	5.2 ± 0.3^{ac}	6.1 ± 0.4^{ab}	6.9 ± 0.4^{a}	3.3 ± 1.1^{bc}	4.7 ± 0.6^{ac}	3.6 ± 0.8^{bc}	3 ± 0.7^{cB}	4.3 ± 0.8^{ac}
Beat cross frequency (Hz)	0h	28.5 ± 2.5	27.8 ± 0.9	27.2 ± 1	27.6 ± 1.3	27 ± 1	30.3 ± 1.5	30.7 ± 1.3	29.9 ± 1.2	30.8 ± 1.3	30.8 ± 1
	12h	28.5 ± 3.3	30.6 ± 1.1	29.7 ± 1	29.5 ± 1	29.8 ± 1.1	29.8 ± 1.6	29.3 ± 1.7	29.4 ± 3.7	27 ± 2.8	29.6 ± 1
	24h	30.8 ± 1	29.8 ± 1.6	31.2 ± 1.1	31.3 ± 0.6	28.3 ± 1.9	25.6 ± 3	26.5 ± 3.5	24.5 ± 3.7	30.2 ± 2.1	23.1 ± 4.2
	36h	26.6 ± 0.8	26 ± 3.5	32.8 ± 1.2	27.3 ± 1.2	29.2 ± 2	27.7 ± 5	25.6 ± 4.6	25.3 ± 2.6	21.3 ± 2.9	26.1 ± 2.1
Straightness (%)	0h	60.1 ± 2.5	61.4 ± 2	59.7 ± 2.4	59.6 ± 2.3	27 ± 1	54.8 ± 2.9	54.7 ± 1.8	53.9 ± 2	30.8 ± 1.3	30.8 ± 1
	12h	44.4 ± 1.7	53.6 ± 2.9	51.3 ± 0.9	53.5 ± 1.3	49.3 ± 1.9	56.2 ± 3.8	53.1 ± 3.5	55 ± 2	54.4 ± 3.5	52.2 ± 1.9
	24h	52.6 ± 1.7	51.8 ± 1.6	51.8 ± 1.5	52 ± 1.1	50.6 ± 1.8	52 ± 2.5	55.7 ± 3.1	59.7 ± 2.7	54.5 ± 3.1	49.1 ± 3.2
	36h	55.2 ± 2	55.4 ± 2	51.3 ± 1.6	53.2 ± 1.7	54.1 ± 2.9	51.2 ± 2.8	59.6 ± 1.8	59.5 ± 3	58.4 ± 2.6	56.4 ± 1.6
Linearity (%)	0h	27.8 ± 1.3	29.9 ± 1	28.6 ± 0.9	27.4 ± 1.1	27.1 ± 1.2	26.2 ± 1.3	26.3 ± 0.6	26.8 ± 1.3	24.8 ± 1^{A}	25 ± 1.2^{A}
	12h	27.7 ± 1	31.8 ± 1.8	29 ± 1	29.5 ± 1.1	26.5 ± 1.2	28.4 ± 1.7	31.4 ± 2.8	30.7 ± 1.7	29.7 ± 2^{AB}	29.7 ± 1.4^{AB}
	24h	29.3 ± 1.3^{ab}	29.9 ± 1.3^{ab}	29.7 ± 0.9^{ab}	28.8 ± 1^{ab}	28.1 ± 1.6^{ab}	28.9 ± 1.4^{ab}	32.6 ± 1.7^{ab}	35.4 ± 2.2^{a}	31.3 ± 2.1^{abAB}	25.2 ± 1.8^{bAB}
	36h	30 ± 1.5	32 ± 1.6	29.3 ± 1.1	29.6 ± 1.2	29.6 ± 1.7	28.1 ± 1.9	34.1 ± 2	33.2 ± 1.9	34.2 ± 2.3^{B}	33.8 ± 2.3^{B}
Medium motility (%)	0h	13.3 ± 3.5	16.6 ± 2	13.9 ± 2.6	13.2 ± 2.6	10.9 ± 3	18.2 ± 3.9	18.6 ± 4.7	15.5 ± 4.8	16.7 ± 3.7	17.2 ± 4.5
	12h	16.7 ± 4.3	13.5 ± 5.7	12.4 ± 2.2	12.1 ± 2	11.2 ± 2.7	7 ± 3.6	6.7 ± 2.7	7.3 ± 3	6.6 ± 2.4	6.6 ± 2.3
	24h	13 ± 3.4	7.6 ± 2	11.4 ± 2.5	11.4 ± 2.9	10.3 ± 2.9	4.1 ± 1.4	3.1 ± 0.8	4.9 ± 1.8	3.9 ± 1	2.7 ± 0.7
	36h	8.3 ± 1.5	4.2 ± 1.1	9.3 ± 2.2	5.4 ± 1.4	7.3 ± 1.6	5.4 ± 2.3	3.9 ± 2.2	2.2 ± 0.4	3.2 ± 2	2.2 ± 0.7
Slow motility (%)	0h	4 ± 0.8	5.5 ± 1.1	4.1 ± 0.6	3.4 ± 0.6	3.6 ± 0.8	5 ± 1.2	4.8 ± 1	4.2 ± 0.8	4.4 ± 0.7	4.7 ± 0.8
	12h	7.8 ± 1.2^{a}	6.2 ± 1.4^{ab}	7.8 ± 1^{a}	7.3 ± 1.4^{ab}	6.8 ± 0.5^{ab}	2.2 ± 0.6^{b}	3.3 ± 0.7^{ab}	2.8 ± 1^{ab}	3.3 ± 0.8^{ab}	3.1 ± 0.5^{ab}
	24h	6.7 ± 1.4^{ab}	4.4 ± 1.2^{ab}	6.7 ± 1.4^{ab}	7.3 ± 1.2^{a}	5.4 ± 0.6^{ab}	2.8 ± 0.8^{ab}	2.1 ± 0.5^{ab}	2.2 ± 0.6^{ab}	2.4 ± 0.7^{ab}	1.8 ± 0.6^{b}
	36h	6.9 ± 1.3	3.2 ± 0.8	6.7 ± 1.3	4.8 ± 1.8	5.1 ± 0.7	2.9 ± 1	2.8 ± 0.7	2 ± 0.3	2.1 ± 0.6	1.6 ± 0.7

$^{a-c}$ Values are expressed as mean ± SEM with different letters in rows differ significantly ($P < 0.05$).

$^{A-B}$ Values with different letters in columns differ significantly between times of the same parameter ($P < 0.05$).

CONCLUSÕES

1. Demonstrou-se a ocorrência de várias bactérias Gram-positivas contaminantes no sêmen e prepúcio de catetos, com destaque para *Corynebacterium* spp. e *Staphylococcus* spp.; bem como um impacto negativo de cargas elevadas de *Corynebacterium* spp. sobre a função espermática no sêmen fresco.

2. A maioria das bactérias isoladas, tanto do sêmen quanto do prepúcio de catetos, foram sensíveis à penicilina-estreptomicina e à gentamicina nas concentrações testadas, enquanto um menor número delas foi sensível ao gel da *Aloe vera*.

3. A combinação penicilina-estreptomicina ou a gentamicina podem ser adicionadas em diluentes de sêmen, incubados a 37 °C por até 180 min ou 120 min, respectivamente, sem causar efeitos tóxicos aos espermatozoides dos catetos.

4. A combinação penicilina-estreptomicina e a gentamicina foram eficazes no controle bacteriano durante a refrigeração do sêmen desses animais, não afetando a longevidade espermática das amostras.

5. O gel da *Aloe vera* não promoveu ação antimicrobiana eficaz durante a refrigeração do sêmen.

PERSPECTIVAS

O primeiro trabalho, que foi realizado por meio da caracterização dos tipos bacterianos do sêmen e prepúcio dos catetos, além do perfil de sensibilidade das cepas frente a substâncias antibacterianas comumente utilizadas na conservação de sêmen, serviu para nortear os estudos seguintes a respeito das drogas e concentrações adequadas. Assim, foi possível a execução das etapas seguintes referentes a avaliação dos efeitos tóxicos no sêmen pelo teste de termo resistência em um curto prazo de tempo, bem como a eficácia dessas drogas como aditivos no diluente para refrigeração.

Os resultados demonstraram que a microbiota dos animais criados em cativeiro é constituída principalmente por microrganismos contaminantes que estão presente na pele e mucosas dos animais e do homem, bem como no ambiente. Uma vez que os resultados para conservação do sêmen foram favoráveis, o presente estudo contribuiu para o aperfeiçoamento da tecnologia de refrigeração do sêmen na espécie, além de ser um trabalho que direciona estudos futuros com viés de controle bacteriano na execução de outras biotécnicas como a criopreservação. Além disso, as informações aqui geradas podem ser também utilizadas durante a execução de biotecnologias reprodutivas que se utilizem do sêmen líquido, como inseminação artificial e fertilização *in vitro*, podendo ser destinadas aos animais criados em cativeiro e extrapoladas para aqueles de vida livre. Porém, orienta-se estudos mais aprofundados sobre a aplicabilidade do sêmen contendo antibióticos vislumbrando a interferência deste em outros aspectos como a fertilidade.

ANEXOS

ANEXO A – Resumo: Análise microbiológica do sêmen e prepúcio de catetos (*Pecari tajacu* Linnaeus, 1758) criados em cativeiro no bioma Caatinga

ANÁLISE MICROBIOLÓGICA DO SÊMEN E PREPÚCIO DE CATETOS (*Pecari tajacu* LINNAEUS, 1758) CRIADOS EM CATIVEIRO NO BIOMA CAATINGA

MICROBIOLOGICAL ANALYSIS OF SEMEN AND FORESKIN FROM COLLARED PECCARIES (*Pecari tajacu* LINNAEUS, 1758) BRED UNDER CAPTIVITY IN CAATINGA BIOME

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Resumo:

O estudo da flora bacteriana do sêmen de catetos é relevante do ponto de vista da tecnologia de sêmen e da sanidade, pois a presença de micro-organismos no ejaculado pode interferir na qualidade espermática e constituir um risco para a disseminação de doenças via inseminação artificial. O objetivo do estudo foi caracterizar a composição bacteriana do sêmen de catetos criados em cativeiro no bioma caatinga. Foram utilizados 5 animais mantidos no Centro de Multiplicação de Animais Silvestres (CEMAS) da Universidade Federal Rural do Semi-Árido (UFERSA). As coletas ocorreram de setembro a outubro de 2016, sendo os animais submetidos a protocolos anestésico e de eletroejaculação. O sêmen foi coletado em frascos esterilizados, sendo precedida a coleta das amostras da mucosa prepucial utilizando-se de suabes esterilizados. As amostras foram acondicionadas em caixa isotérmica e encaminhadas para análises no Laboratório de Microbiologia Veterinária da UFERSA. Cada amostra de sêmen foi submetida a uma diluição seriada até 10^5 para quantificação bacteriana. Posteriormente, alíquotas de cada diluição, bem como os suabes do prepúcio, foram semeados em ágar sangue de carneiro desfibrinado a 5% e ágar MacConkey, em duplicata. Após incubação (37°C/24h), as colônias foram isoladas e identificadas. Os dados foram analisados mediante estatística descritiva. Os resultados demonstraram que o gênero de bactéria mais frequente no sêmen *in natura* foi o *Corynebacterium* spp. (74%), seguida de *Staphylococcus* spp. (17%) e *Arcanobacterium* spp. (9%), com médias

ANEXO B – Resumo: Adição de gentamicina ao diluente reduz a carga bacteriana e não afeta a qualidade do sêmen refrigerado de catetos (*Pecari tajacu*)

Congresso Brasileiro de Reprodução Animal, 23, 2019, Gramado, RS. *Anais*... Belo Horizonte: CBRA, 2019.

Adição de gentamicina ao diluente reduz a carga bacteriana e não afeta a qualidade do sêmen refrigerado de catetos (*Pecari tajacu*)

Addition of gentamicin to the extender decrease the bacterial load and does not affect the quality of collared peccary (Pecari tajacu) semen

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Apesar da necessidade de inclusão de agentes antimicrobianos nos diluentes para sêmen com o intuito de controlar a proliferação de microrganismos, é do conhecimento científico que tal procedimento pode resultar tanto em efeitos benéficos como tóxicos. Visando a conservação de germoplasma de animais silvestres, o desenvolvimento de biobancos para tais espécies tem sido constantemente fomentado. Neste sentido, este trabalho objetivou avaliar o efeito da inclusão da gentamicina no diluente para o sêmen de catetos sobre a carga bacteriana e os parâmetros espermáticos das amostras submetidas a refrigeração. Para tanto, foram utilizados cinco animais adultos mantidos no Centro de Multiplicação de Animais Silvestres (CEMAS) da Universidade Federal Rural do Semi-Árido (UFERSA). As coletas ocorreram de maio a junho de 2018, sendo os animais submetidos à anestesia com propofol (5mg/Kg) por via intravenosa em bolus e eletroejaculação. O sêmen coletado foi diluído em Tris-frutose-ácido cítrico acrescido de 20% de gema de ovo, dividido em três alíquotas, sendo a primeira referente ao controle sem antibiótico e duas acrescidas de gentamicina (30µg/mL - G30 ou 70µg/mL - G70). As amostras foram mantidas refrigeradas a 5°C por até 36 horas, sendo avaliadas imediatamente após a diluição e depois a cada 12 horas (0, 12, 24 e 36h). Em cada tempo, foram realizadas avaliações da motilidade total (MT) por análise computadorizada (Sistema IVOS, Hamilton-Thorne, Estados Unidos), e da integridade da membrana (IM) por meio de sondas fluorescentes (Hoescht, Iodeto de Propídio). Paralelamente, foi realizada a quantificação de bactérias mesófilas (UFC/mL) por meio da técnica de contagem em placas. As médias dos resultados foram analisadas pelo teste t ($P<0,05$). Em cada período de refrigeração, os percentuais médios de motilidade total e integridade de membrana não diferiram ($p<0,05$) entre os tratamentos com gentamicina e o controle. Os valores médios e erros padrão para motilidade após 36 horas foram de 19,6 ± 9,5%, 27,8 ± 9,4%, e 27 ±10,2% para os tratamentos G70, G30 e controle, respectivamente. Já os percentuais médios de espermatozoides com membranas integras após 36 horas foram de 59 ± 3,8%, 62,2 ± 3,5% e 62,4 ± 4,4% para os tratamentos G70, G30 e controle, respectivamente. As amostras de sêmen diluídas com a maior concentração de gentamicina (G70) apresentaram menor carga bacteriana ($P<0,05$) do que o controle logo após a diluição (0h), bem como após 12 e 36 horas de refrigeração. As contagens médias, em UFC/mL, foram de $2,2 \pm 1 \times 10^4$, $3,9 \pm 2,3 \times 10^4$ e $1,3 \pm 0,78 \times 10^4$ para o tratamento G70 às 0, 12 e 36 horas, respectivamente. As contagens médias para o tratamento controle, nos mesmos tempos de refrigeração, foram de $2,4 \pm 0,82 \times 10^5$, $4,6 \pm 1,5 \times 10^5$ e $2,3 \pm 0,91 \times 10^5$ UFC/mL, respectivamente. Conclui-se que a adição de gentamicina na concentração de 70µg/mL possibilita o controle bacteriano na amostra, bem como não apresenta efeito tóxico sobre a qualidade do sêmen refrigerado de catetos mantido por até 36 horas.

Palavras-chave: biobanco, espermatozoide, *Tayassu tajacu*.
Keywords: *biobank, sperm, Tayassu tajacu.*

ANEXO C – Resumo: Efeito da inclusão de penicilina-estreptomicina no diluente para refrigeração do sêmen de catetos (*Pecari tajacu*)

Congresso Brasileiro de Reprodução Animal, 23, 2019, Gramado, RS. *Anais*... Belo Horizonte: CBRA, 2019.

Efeito da inclusão de penicilina-estreptomicina no diluente para refrigeração do sêmen de catetos (*Pecari tajacu*)

Effect of the penicillin-streptomycin inclusion on the extender for collared peccary (Pecari tajacu) semen cooling

Caio Sérgio Santos, Maiko Roberto Tavares Dantas*, Marina Crisley Gondim Rebouças, Andréia Maria da Silva, Érica Camila Gurgel Praxedes, Lívia Batista Campos, Moacir Franco Oliveira, Alexandre Rodrigues Silva

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Apesar de necessária sua inclusão nos diluentes de sêmen, alguns antibacterianos, como a associação penicilina-estreptomicina, podem apresentar efeitos tóxicos a depender de sua concentração. Assim, esse trabalho objetivou avaliar o efeito da inclusão de penicilina-estreptomicina sobre a carga bacteriana, motilidade e integridade de membrana de espermatozoides de catetos (*Pecari tajacu*) durante a refrigeração de sêmen. Foram utilizados cinco animais mantidos no Centro de Multiplicação de Animais Silvestres (CEMAS) da Universidade Federal Rural do Semi-Árido (UFERSA). As coletas ocorreram de maio a junho de 2018, sendo os animais submetidos à anestesia e eletroejaculação. O sêmen coletado foi diluído em Tris-citrato-frutose + 20% de gema de ovo, dividido em três alíquotas, sendo uma controle e duas acrescidas de penicilina-estreptomicina (2000 UI e 2mg/mL - P+E 2000/2 e 1000 UI e 1 mg/mL - P+E 1000/1). As amostras foram mantidas refrigeradas a 5°C por até 36 horas, sendo avaliadas imediatamente após a diluição e depois a cada 12 horas (0, 12, 24 e 36h). Em cada tempo, foram realizadas avaliações da motilidade total (MT), por análise computadorizada (sistema IVOS), da integridade da membrana (IM) por meio de sondas fluorescentes (Hoescht/ Iodeto de propídio), bem como a quantificação de bactérias mesófilas (UFC/mL) por meio da técnica de contagem em placas. As médias dos resultados foram analisadas pelo teste t de Student ($P<0,05$). Os percentuais médios de motilidade total e integridade de membrana não diferiram ($P>0,05$) entre os tratamentos com P+E e o controle durante cada período de refrigeração. Os valores médios (±EP) para motilidade após 36 horas foram de 9,6 ± 3,0, 22,8 ± 8,5, 27 ± 10,2 para os tratamentos P+E 2000/2, P+E 1000/1 e controle, respectivamente. Já os percentuais médios de espermatozoides com membranas integras após 36 horas foram de 48,8 ± 6,7, 62,2 ± 4,34 e 62,4 ± 4,4 para os mesmos tratamentos, respectivamente. As amostras de sêmen diluídas com penicilina e estreptomicina apresentaram menor carga bacteriana ($P<0,05$) do que o controle logo após a diluição (0h), bem como com 12 e 36 horas de refrigeração. As contagens médias, em UFC/mL, foram de $1,1 \pm 0,7 \times 10^3$, $2,0 \pm 1,2 \times 10^3$ e $3,2 \pm 3,0 \times 10^3$ para o tratamento P+E 2000/2 às 0, 12 e 36 horas respectivamente. As contagens médias, em UFC/mL, foram de $1,5 \pm 1,1 \times 10^4$, $2,7 \pm 2,5 \times 10^4$ e $1,2 \pm 0,8 \times 10^4$ para o tratamento P+E 1000/1 às 0, 12 e 36 horas, respectivamente. E as contagens médias para o tratamento controle, nos mesmos tempos de refrigeração, foram de $2,4 \pm 0,8 \times 10^5$, $4,6 \pm 1,5 \times 10^5$ e $2,3 \pm 0,9 \times 10^5$ UFC/mL, respectivamente. Conclui-se que, a associação penicilina-estreptomicina, em qualquer das concentrações testadas, pode ser eficientemente utilizada na refrigeração do sêmen de catetos, haja vista não apresentar toxicidade sobre os parâmetros testados e ser capaz de controlar a carga bacteriana nas amostras.

Palavras-chave: antimicrobiano, biobanco, animal selvagem.
Keywords: *antimicrobial, biobank, wild animal.*

ANEXO D – Carta de submissão de artigo: Journal of Applied Microbiology

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28-Jan-2020

Dear Dr. Silva

The following manuscript has been successfully submitted to Journal of Applied Microbiology.

Manuscript ID: JAM-2020-0153
Title: Characterization of semen and foreskin microbiota and its impact on sperm parameters of captive collared peccaries (Pecari tajacu)
Authors: Santos, Caio Sérgio; Silva, Andreia; Maia, Keilla; Rodrigues, Gardenia; FEIJO, FRANCISCO; Alves, Nilza; Oliveira, Moacir; Silva, Alexandre

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Corresponding author: Dr Alexandre Silva
Listed co-author(s): Dr Caio Santos, Dr Lívia Campos, Mrs Erica Praxedes, Miss Samara Moreira, Miss Marina Rebouças, Dr João Batista Freire Souza-Junior, Dr Francisco Marlon Feijó

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